JOURNAL OF
GENERAL VIROLOGY

SHORT COMMUNICATION
Marschall *et al.*, *Journal of General Virology*
DOI 10.1099/jgv.0.001370

MICROBIOLOGY SOCIETY

Differential upregulation of host cell protein kinases by the replication of α-, β- and γ-herpesviruses provides a signature of virus-specific signalling

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Abstract

Infections with human herpesviruses share several molecular characteristics, but the diversified medical outcomes are distinct to viral subfamilies and species. Notably, both clinical and molecular correlates of infection are a challenging field and distinct patterns of virus–host interaction have rarely been defined; this study therefore focuses on the search for virus-specific molecular indicators. As previous studies have demonstrated the impact of herpesvirus infections on changes in host signalling pathways, we illustrate virus-modulated expression levels of individual cellular protein kinases. Current data reveal (i) α-, β- and γ-herpesvirus-specific patterns of kinase modulation as well as (ii) differential levels of up-/downregulated kinase expression and phosphorylation, which collectively suggest (iii) defined signalling patterns specific for the various viruses (VSS) that may prove useful for defining molecular indicators. Combined, the study confirms the correlation between herpesviral replication and modulation of signalling kinases, possibly exploitable for the *in vitro* characterization of viral infections.

INTRODUCTION

Human and animal herpesviruses show distinct molecular patterns of virus–host interaction. Herpesviruses are worldwide distributed pathogens of humans and animals that cause a variety of clinical symptoms and diseases distinct for each individual virus species. As far as the prototypes of the α-, β- and γ-herpesviruses subfamilies are concerned, i.e. herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV), the range of clinical manifestations can vary substantially. Disease following HSV-1 infection can extend from mild illnesses such as cold sores, easily tolerable in most individuals, to sporadic, life-threatening neurological devastation during the pathogenesis of HSV encephalitis [1]. HCMV disease manifestations can range from self-limiting febrile periods to fatal end-organ disease. Specifically, congenital HCMV infection acquired during pregnancy still represents a serious medical problem, frequently leading to severe developmental defects and life-threatening cytomegalovirus inclusion disease. HCMV reactivation and reinfection may occasionally occur, both mostly asymptomatic or accompanied by mild febrile illness. In immunosuppressed individuals, however, HCMV infection can lead to severe symptoms, and HCMV itself may further weaken immune responses [2, 3]. EBV infection can lead to acute infectious mononucleosis, which is normally self-limiting, yet EBV is also associated with a number of human cancers such as Burkitt's lymphoma, nasopharyngeal carcinoma, post-transplant B and T cell lymphomas, and

Received 04 November 2019; Accepted 26 November 2019; Published 20 January 2020
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Keywords: herpesviral replication; primary human fibroblasts; modulation of the host kinome; differential upregulation of cellular kinases; signature of virus-specific signalling.
Abbreviations: Ad5, adenovirus 5; CDK, cyclin-dependent kinase; EBV, Epstein–Barr virus; HCMV, human cytomegalovirus; HFFs, human foreskin fibroblast; HHV-6A, human herpesvirus type 6A; HSV-1, herpes simplex virus type 1; MHV-68, murine herpesvirus 68; PBMC, peripheral blood mononuclear cell; RhCMV, rhesus monkey cytomegalovirus; SILAC, stable isotope labelling by amino acids in cell culture; VSS, virus-specific signalling; VZV, varicella zoster virus; Wb, Western blot.
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gastric cancer [4]. To date, no vaccines have been approved for the prevention of herpesviral infections, with the exception of varicella zoster virus (VZV)-related diseases. Diagnostic tools and antiherpesviral drugs are available, but in many specific cases they are problematic in terms of limited drug compatibility due to adverse side-effects and thus have remained unsatisfactory to date, so that the repertoire used to characterize viral infections and predictors of disease still awaits further improvement. Infections with human and animal herpesviruses not only exploit cellular metabolic resources for the benefit of viral replication, but also induce instructive molecular patterns of virus–host interaction. In particular, the virus-specific interference with the cellular kinome, as effected through the up- and downregulation of regulatory protein kinases involved in cellular signalling, has been increasingly recognized [5–7]. A number of previous studies have stressed the strong impact of herpesvirus infections on changes of such parameters, i.e. modulated expression levels, phosphorylation status and activity of individual signalling-specific protein kinases. As an example, our studies based on several different methods analysing HCMV-infected primary human foreskin fibroblasts (HFFs) [7–13] have underlined this statement and illustrated for a panel of prominent protein kinases, mostly serine/threonine-specific (with the exception of the tyrosine-specific ABL2 kinase), that these are subject to substantial quantitative upregulation compared to mock-infected cells (Table S1, available in the online version of this article). Here, an interesting feature is the strong viral modulatory impact onto the group of cyclin-dependent kinases (CDKs) and cyclins, which induces a drastic change in the CDK–cyclin expression pattern, known as the phenomenon of virus-induced pseudomitosis [14]. To further address these issues, we performed experiments to analyse the expression characteristics of selected host cell protein kinases during the replication of herpesviruses in fibroblasts and lymphoid cells.

Comparative analysis of herpesviruses from three subfamilies suggests signatures of virus-specific signalling (VSS) based on differential modulation of cellular kinases

The pronounced regulatory capacity of herpesviruses directed to host cell protein kinases was exemplified by the analysis of a selection of viruses belonging to the three subfamilies α (HSV-1; VZV), *β* (HCMV; HHV-6A, human herpesvirus type 6A; RhCMV, rhesus monkey cytomegalovirus) and γ (MHV-68, murine herpesvirus 68; EBV), respectively. A common feature of most of these viruses is their ability to infect HFFs *in vitro*, and using these cells, specific viral host-directed effects have been demonstrated in our previous studies [15, 16]. Here, we addressed the question of how far the modulatory effect onto host kinases, recently identified for HCMV infection as shown by Table S1, is similarly detectable for other herpesviruses. To this end, we infected HFFs with a selection of herpesviruses at increasing m.o.i. values for 3–5 days (HHV-6A infection was achieved by cocultivation of HFFs with HHV-6A-producing J-Jhan cells as previously described [15]). Total lysates were prepared and subjected to standard SDS-PAGE/Western blot (Wb) analysis for immunostaining using a panel of kinase-specific antibodies (Fig. 1). As an important result, a number of analysed cellular protein kinases were upregulated not only by HCMV, but also by other herpesviruses (Fig. 1a). Only one example of downregulation was obtained, namely the tyrosine kinase ABL2 through HSV-1 infection. Interestingly, ABL2 was also modulated by infection with either of the three *β*-herpesviruses, HCMV, HHV-6A or RhCMV, in terms of a change in the expression pattern from one prominent ABL2 band in mock-infected cells towards two different protein varieties, most probably due to a virus-induced alteration in its phosphorylation status. The latter, however, could not be assigned to the known phosphorylation-specific activation site Y412 (which was seen to be upregulated exclusively in HSV-1 samples), but may result from the *β*-herpesvirus-specific conversion of some different phosphorylation sites. Of note, CDKs 1, 2 and 7 as well as several other signalling-relevant kinases, such as Aurora A, p-AMPK (T127) and p-ABL (Y412), showed upregulation in individual virus-infected samples (Fig. 1a; Fig. 1b, upper part), while two kinase stainings did not indicate any modulation, i.e. AMPK and p-PKC (T515) (Fig. 1a; Fig. 1b, lower part). In fact, the findings for AMPK in this and previous studies seem heterogeneous, because a phospho-specific upregulation of AMPK by HCMV is detectable (Fig. 1) [7], while an upregulation of total AMPK expression levels may only sometimes be detectable when using Wb or MS, SILAC (stable isotope labelling by amino acids in cell culture) labelling or other sensitive read-out systems (Table S1) [7, 17]. For those viruses showing poor permissiveness of productive infection in fibroblasts, i.e. HHV-6A and EBV, the respective analysis was extended to T or B cells, respectively (Fig. 1c–d). Interestingly, within a selection of kinases considered relevant in the previous experiments, the two cyclin-dependent kinases CDK2 and CDK7 showing responses of upregulation after *β*- and/or γ-herpesviral infections in fibroblasts, did not induce similar changes in lymphoid cells, possibly due to the high levels of constitutive CDK expression. ABL2 levels were likewise found to be unaltered by infection, whereas Aurora A kinase was upregulated by HHV-6A but not by EBV, similar to the signals obtained for *β*- and γ-herpesviruses in HFFs. The pattern detected for p-PKC (T514) after infection of lymphoid cells with these two viruses was more complex and even varied between individual experiments (data not shown). While productive HHV-6A infection of T cells showed signs of phosphorylation-specific upregulation (albeit in a manner independent of m.o.i.), EBV infection of B cells indicated downregulation (Fig. 1c–d, lower panels). It is known that PKC activation is a multistep process, during which phosphorylation of the activation loop threonine 514 residue is only one of several steps, individually not clearly defined in terms of activity. In addition, the applied phosphorylation-specific antibody mainly recognizes T514 of PKC isoform γ (plus the corresponding residues of some other isoforms), while EBV reactivation primarily correlates with PKCδ coactivation, so that this detection pattern was not considered as a reliable indicator of herpesviral infection in lymphoid cells. Seen apart from this latter limitation, the

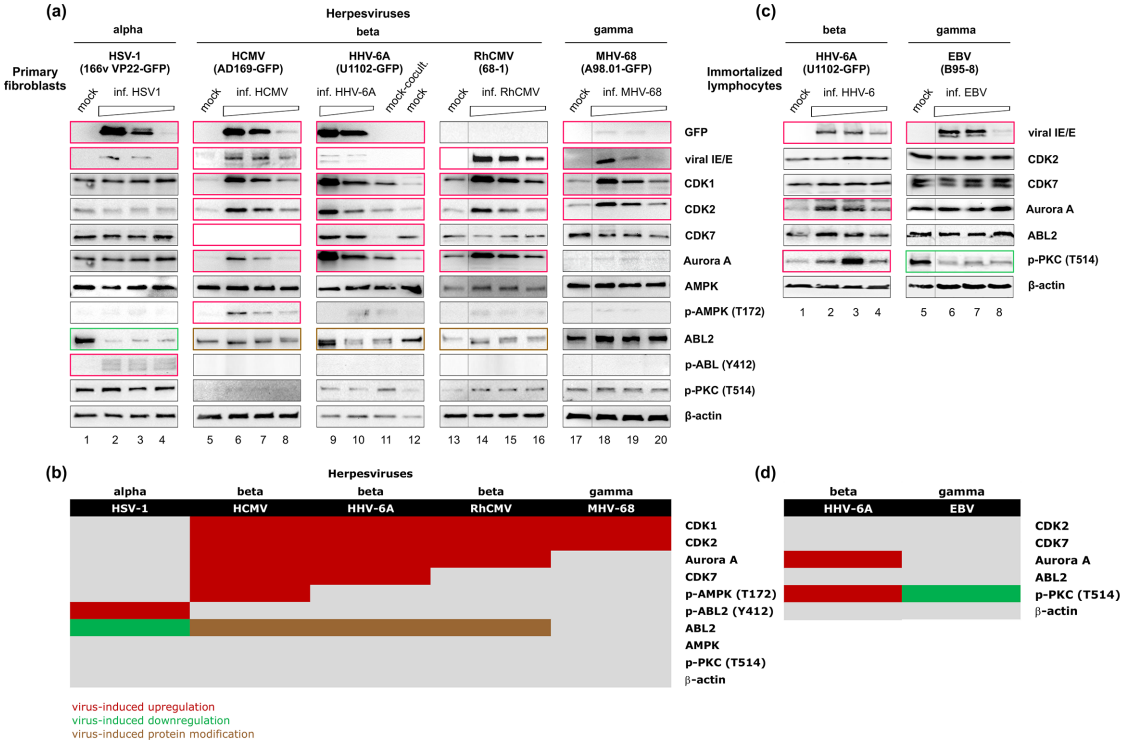

Fig. 1. Virus-specific modulation of the expression levels of selected host cell protein kinases. Primary human foreskin fibroblasts (HFFs) were grown in minimal essential medium (MEM; Gibco) supplemented with 7.5% (v/v) FCS (Sigma-Aldrich), 10 µg ml^{-1} gentamicin, and 350 µg ml^{-1} glutamine, and used for the infection at three different m.o.i. values (approx. 1, 0.3 and 0.1) with herpesviruses from the subfamilies indicated. Cells were harvested 3–5 days post-infection, and total cell lysates were prepared in denaturating SDS loading buffer and subjected to standard SDS-PAGE/Western blot (Wb) procedures. Wb data were derived from immunostaining using antibodies detecting viral marker proteins, cellular protein kinases and phosphorylation-activated kinase versions as described in the supporting data. (a) Virus strains and recombinants used (coloured frames as described below): HSV-1, 166 v VP22-GFP; HCMV, AD169-GFP; HHV-6A, U1102-GFP; RhCMV, 68-1; MHV-68, A89.01-GFP [15]. (b) Schematic depiction summarizing the graduated extent of virus-induced changes of selected protein kinases, i.e. a quantitative upregulation (red; upper part), downregulation (green; middle part), a qualitative modification, i.e. a shift in size of the detectable protein varieties (brown; middle part) or unaltered through virus infection (grey, lower part).

findings in essence suggest a pronounced and reproducible signature of VSS, which should be useful as a tool for the characterization of herpesvirus-specific *in vitro* infections, but which still may require further elaboration using additional methods.

Specific features of the herpesviral VSS patterns

An interesting property of α-herpesviral upregulation was identified for the multiregulatory autophagy-associated host kinase ULK1. When analysing serial m.o.i. values of HSV-1 and VZV infection in parallel (VZV infection was achieved through cocultivation with virus-positive HFFs as inoculum) [18], no drastic quantitative changes in the overall expression levels of ULK1 were observed. However, specific qualitative alteration was noted, in terms of strongly upregulated phosphosites Ser317, Ser556, Ser638 and Ser758, but exclusively through HSV-1 not VZV infection (Fig. 2). This clearly illustrated profound differences in VSS between viruses, even among two related viruses within one herpesviral subfamily. As an example, more obviously referring to the natural infection situation, we used peripheral blood mononuclear cells (PBMCs) of diagnostically characterized donors with variable EBV seropositive/seronegative status for the Wb-based kinase analysis. EBV infection status was determined diagnostically by the Immunoblot EBV IgG recomLine assay (Mikrogen), which detects antibodies to EBV antigens EBNA1, VCA-p18, VCA-p23, BZLF1, EA-p54 and EA-p138. In parallel, anti-EBV IgG and IgM were measured using Enzygnost EBV IgG/IgM ELISA (Siemens Healthcare Diagnostics). The Wb results appear noticeable given that CDK1, but not CDK7, upregulation was exclusively detected in two out of four sample donors with an EBV-associated infectious mononucleosis (IM) (Fig. S1; expression of EBV kinase BGLF4 could not be detected, due to the low number of EBV-positive cells). It should be stressed, however, that these initial data produced with clinical PBMC samples containing a mixture of cell types do not allow specific conclusions to be made on the nature of kinase-inducing stimuli. At this stage, we cannot exclude the possibility that a variety of CDK1-inducing stimuli, other than EBV, might have produced such

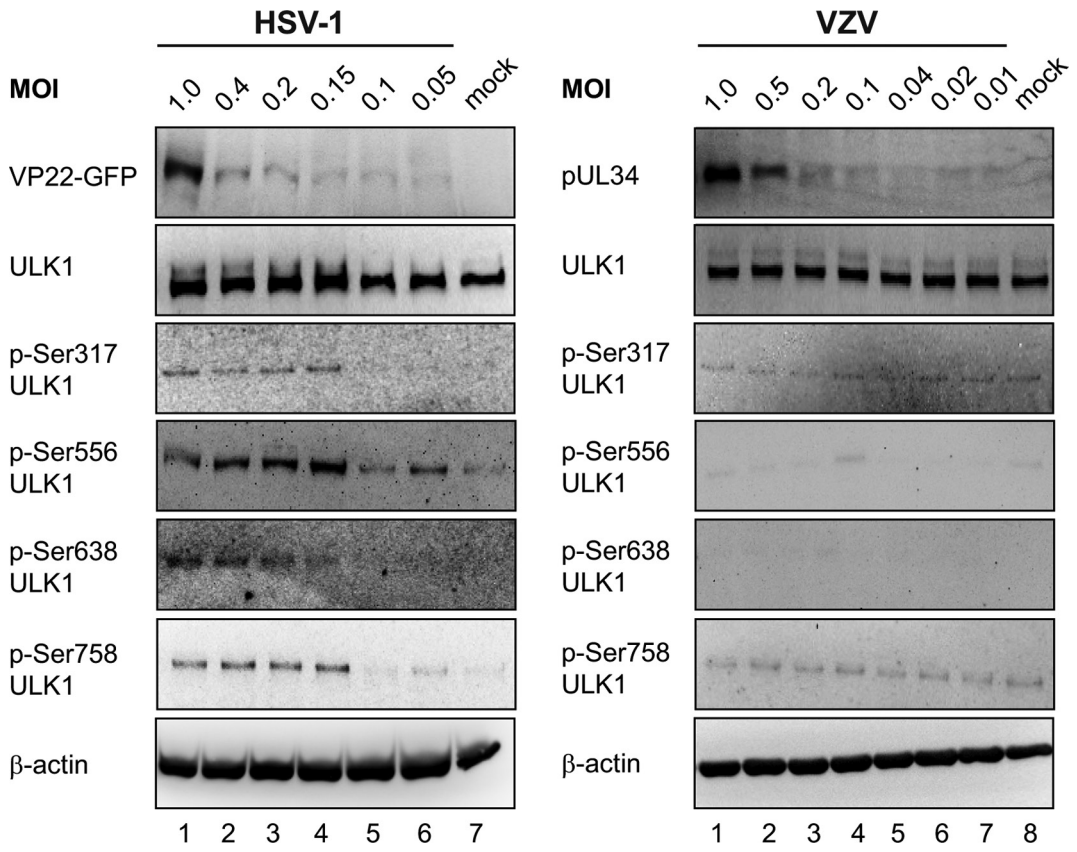

Fig. 2. Virus-specific modulation of the phosphorylation levels of the host ULK1 protein kinase. HFFs were infected with HSV-1 (166v VP22-GFP, left panels) or VZV (vaccine strain Oka, right panels) at the indicated m.o.i. values. HSV-1-infected HFFs were harvested 22 h post-infection (p.i.), VZV-infected HFFs 5 days p.i., and total cell lysates were prepared in denaturating SDS loading buffer and subjected to standard SDS-PAGE/Wb procedures. Immunostaining was performed against the indicated protein varieties using antibodies described in the supporting data.

detectable upregulation. On this basis, the current setting may rather be considered as a long-term option to address VSS analysis using a larger number of materials, possibly also including cell-type-sorted clinical samples and applying more sensitive methods for the detection of kinase upregulation, such as MS. Finally, we addressed whether the exposure of cells to individual herpesviral proteins may already lead to detectable changes in intracellular kinase levels. HCMV was one of the viruses showing the most drastic induction of kinase-specific alterations. Cytomegaloviral immediate early and tegument proteins were considered as putative regulatory candidates, as they are involved in virus-supportive events of host interaction, such as the modulation of nuclear bodies, cell cycle components and signalling [14, 19, 20]. As two specific proteins of interest, we chose the regulatory HCMV proteins IE1p72 and pp65, because the first is known to exert strong effects on PML nuclear bodies [19], the second as a coregulator of host innate immunity [21, 22], also eventually uptaken from the external environment by cells showing pp65 antigenaemia [23]. These proteins were either topically expressed through an adenovirus 5 (Ad5) vector [24] or through extracellular incubation of purified viral protein in culture [25]. Notably, in IE1p72, neither the Ad5-mediated expression nor the addition of external protein alone could mimic the demonstrated HCMV infection-induced effects on CDK1, CDK2, Aurora A or ULK1 (Fig. 3a, b, central panel). Similarly, external addition of pp65 did not show upregulation of CDK1, CDK7, ABL2 or Aurora A (Fig. 3b, central panel; Fig. 3c). Interestingly, such kinase-specific effects were missing even though some partial uptake of pp65 into HFFs could be demonstrated by confocal imaging (Fig. 3d) and even though immunological activity of the purified protein was demonstrated by respective assays [23] (R.K., R.W., personal communication). These findings further support our concept that intracellular replication of individual herpesviruses (but not necessarily the extracellular/intracellular presence of a single viral protein) can exert a strong, sometimes virus-specific effect on the host proteome, as specifically illustrated for protein kinases.

Conclusions

This study was based on the search for distinct characteristics of herpesvirus–host interactions, which were classified

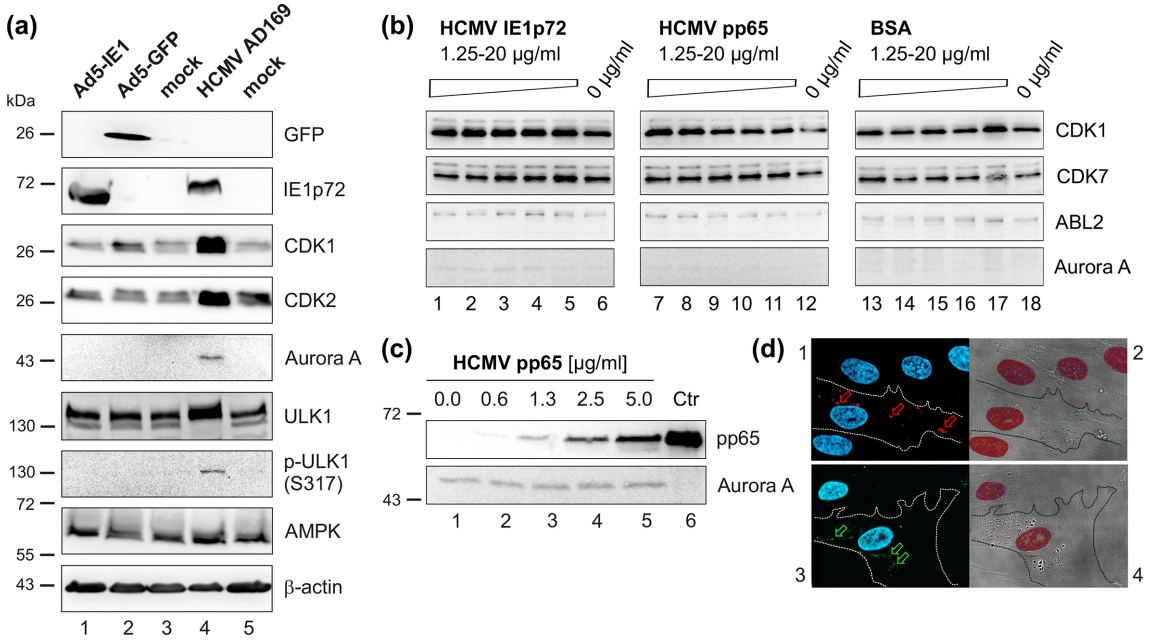

Fig. 3. Putative effect of HCMV IE1p72 or pp65, expressed by a recombinant Ad5 vector or externally added as purified proteins, onto HFFs cultivated in six-well plates. Subconfluent layers of HFFs were used to transduce Ad5-IE1 (Ad5-GFP as a control) or to incubate the indicated amounts of purified proteins in culture supernatants for the duration of 3 days (GST-tagged IE1p72 was affinity-purified from recombinant expression in *Escherichia coli*, whereas His-tagged pp65 or GFP-pp65 were purified from recombinant baculovirus expression in insect cells; see the supporting data). Thereafter, cells were harvested, and total cell lysates were prepared in denaturating SDS loading buffer and subjected to standard SDS-PAGE/Wb procedures. Immunostaining was performed against the indicated proteins using antibodies as described in the supporting data. (a) Ad5-mediated transduction experiment with HFFs. Infection with HCMV strain AD169, m.o.i. 1, was performed as a positive control; mock-infected HFFs were used as negative controls. (b) HFFs were incubated with IE1p72, pp65 or control protein BSA (Sigma) at concentrations of 1.25, 2.5, 5, 10, 20 and 0 µg ml^{-1}. (c) Using an identical setting, the residual pp65 was immunostained on Western blots derived from the SDS-PAGE separation of total cell lysates, in parallel to the Aurora A staining (a sample of the input pp65 was loaded separately, lane 6, as a staining control, CTR). (d) Intracellular uptake of pp65 (red or green arrows) was demonstrated by immunofluorescence staining and confocal imaging using mAb-pp65-mediated staining of pp65, panels 1–2, or direct autofluorescence of GFP-pp65, panels 3–4, according to previous procedures [26]. Panels 1 and 3, fluorescence channel, with DAPI used as a nuclear counterstaining; panels 2 and 4, brightfield channel.

in patterns of VSS kinases and might be relevant for future use in defining virus-specific molecular indicators of *in vitro* infection. Our previous approaches had initially started with analyses of SILAC-labelled or label-free MS-based proteomics using material from cytomegalovirus-infected cells [7, 12, 13], and have then been continued by Wb analyses also including phospho-specific antibodies and radioactive *in vitro* kinase assays [8, 10, 11, 13]. Combined, our findings confirm the correlation between herpesviral replication and the modulation, in most cases upregulation, of cellular protein kinases. As an outlook perspective, the continuation of this approach might also be applicable for the analysis of broader panels of infected-cell material and the characterization of virus-specific indicator proteins.

Funding information
The study was supported by grants from Bayerische Forschungsstiftung (Forschungsverbund 'ForBIMed–Biomarker in der Infektionsmedizin'), Wilhelm Sander-Stiftung (grants 2018.121.1 and 2011.085.2) and Deutsche Forschungsgemeinschaft (MA1289/8-1).

Acknowledgements
We thank Stefan Strobl (BioNTech AG Small Molecules, formerly 4SC Discovery), Zsolt Ruzsics (Institute for Virology, University Medical Center, Freiburg), Ludwig Deml (Lophius Biosciences), Alexander Steinkasserer (Immunomodulation, Experimental Dermatology, FAU, Erlangen), André Gessner and Benedikt Asbach (Institute for Microbiology and Hygiene, UREG, Regensburg) for providing valuable materials and scientific advice.

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

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