Protein kinases responsible for the phosphorylation of the nuclear egress core complex of human cytomegalovirus

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Abstract

Nuclear egress of herpesvirus capsids is mediated by a multi-component nuclear egress complex (NEC) assembled by a heterodimer of two essential viral core egress proteins. In the case of human cytomegalovirus (HCMV), this core NEC is defined by the interaction between the membrane-anchored pUL50 and its nuclear cofactor, pUL53. NEC protein phosphorylation is considered to be an important regulatory step, so this study focused on the respective role of viral and cellular protein kinases. Multiply phosphorylated pUL50 varieties were detected by Western blot and Phos-tag analyses as resulting from both viral and cellular kinase activities. In vitro kinase analyses demonstrated that pUL50 is a substrate of both PKCa and CDK1, while pUL53 can also be moderately phosphorylated by CDK1. The use of kinase inhibitors further illustrated the importance of distinct kinases for core NEC phosphorylation. Importantly, mass spectrometry-based proteomic analyses identified five major and nine minor sites of pUL50 phosphorylation. The functional relevance of core NEC phosphorylation was confirmed by various experimental settings, including kinase knock-down/knock-out and confocal imaging, in which it was found that (i) HCMV core NEC proteins are not phosphorylated solely by viral pUL97, but also by cellular kinases; (ii) both PKC and CDK1 phosphorylation are detectable for pUL50; (iii) no impact of PKC phosphorylation on NEC functionality has been identified so far; (iv) nonetheless, CDK1-specific phosphorylation appears to be required for functional core NEC interaction. In summary, our findings provide the first evidence that the HCMV core NEC is phosphorylated by cellular kinases, and that the complex pattern of NEC phosphorylation has functional relevance.

INTRODUCTION

Human cytomegalovirus (HCMV) is a globally distributed beta-herpesvirus that causes life-long latent infection in the human host. In the immunocompetent host, HCMV may remain asymptomatic, whereas in immunosuppressed individuals, e.g. transplant recipients, those with tumours and AIDS patients, HCMV infection can lead to severe symptoms and life-threatening viral pathogenesis. Most seriously, congenital HCMV infection acquired during pregnancy represents a serious risk for the unborn and neonates to develop severe developmental defects and cytomegalovirus inclusion disease. Viral pathogenesis is closely linked with the efficiency of viral replication in individual tissues, a pronounced virulence, and so far insufficiently understood determinants of virus–host interaction. On the molecular level, recent investigations have stressed the importance of multiprotein complexes assembled between virus and host constituents. Such protein complexes appear to represent rate-limiting determinants of cytomegalovirus replication.

We and others have concentrated on the study of the HCMV-specific nuclear egress complex (NEC). The nuclear envelope represents a physical barrier separating the nucleus from the cytoplasm. HCMV genomic replication starts in the host cell nucleus, where preformed capsids are packaged and exported to the cytoplasm for further virion maturation. The nuclear–cytoplasmic transition of capsids is a
multistep regulatory process, termed nuclear egress. During this process, the nuclear envelope is reorganized at specific sites, with the phosphorylation-triggered distortion of the nuclear lamina being profoundly important. The HCMV-encoded protein kinase pUL97 was identified as the first herpesviral kinase with lamin-phosphorylating activity [1]. Very recent investigations indicated that the recruitment of lamin-phosphorylating protein kinase activity, as well as further lamin-modifying proteins, such as prolyl cis/trans isomerase Pin1, has central importance [2]. A scaffold for the assembly of various NEC-associated enzymes and regulatory factors is provided by two HCMV proteins, pUL50 and pUL53, which heterodimerize and corecruit each other in the form of a rim-like association at the inner side of the nuclear envelope. As a consequence of this core NEC formation in HCMV-infected nuclei, the various proteins of a multi-component NEC are associated in a dynamic fashion [3]. The entity on NEC-associated components induces massive reorganization of the nuclear lamina and triggers a targeted nuclear envelope transition of viral capsids. The phosphorylation of the constituents of herpesviral NECs is considered to be a crucial step for full NEC functionality. It appears to be generally accepted that both viral and cellular protein kinases can be responsible for specific modifications of NEC components, as well as lamins/lamin-associated proteins and, consequently, the phosphorylation-dependent disassembly of the nuclear lamina (Table 1) [1, 4–15]. In the case of HCMV, Sharma et al. showed that pUL50–pUL53 core NEC formation at the nuclear rim is dependent on the activity of the viral kinase, pUL97 [8]. In addition, our recently reported data strongly suggested a similar dependence on cyclin-dependent kinases (CDKs), when CDK inhibitors were analysed, showing in specific cases a nuclear rim dyslocalization of pUL50–pUL53 in HCMV-infected cells [16]. Concerning the dynamic nature of at least three distinct activities of the HCMV core NEC, namely the recruitment of effectors, the induction of nuclear envelope rearrangement and the NEC docking to nuclear capsids [17], a number of recent studies have highlighted the fact that protein phosphorylation is a key regulator of NEC functionality. First and foremost, phosphorylation of the multicomponent NEC mediates a distortion of the lamina through site-specific phosphorylation of nuclear lamins. In particular, the core NEC component pUL50 interacts with at least three protein kinases, either directly [CDK1 and protein kinase C (PKC)] or indirectly (via bridging of pUL97 through p32/gC1qR). Moreover, all of the NEC-associated components that have been characterized to date are phospho-proteins themselves. Primarily, pUL50 and pUL53 are both heavily phosphorylated, but so far little is known about the responsible kinases and the functional consequences. In this study, we provide evidence concerning the phosphorylation patterns of pUL50 and pUL53, and assess the major and minor sites of pUL50 phosphorylation, the putative role of specific protein kinases (pUL97, PKC and CDK1) and the relevance of CDK1-specific phosphorylation for the HCMV core NEC. A refined model of phosphorylation-conferring core NEC functionality is presented.

RESULTS

HCMV core NEC proteins pUL50 and pUL53 can be dually phosphorylated by pUL97 and, additionally, in a pUL97-independent manner

In order to address the question of which protein kinases are involved in HCMV core NEC phosphorylation, 293T cells were transfected with plasmids coding for pUL50 and pUL97 and treated with lambda phosphatase (lambda PP) (Fig. 1a). Coexpression of the two proteins led to higher-molecular-weight bands of pUL50 being visible in immunoprecipitates or in total lysates (Fig. 1a, lanes 3 and 4). However, these additional bands were depleted after incubation with lambda PP, thereby confirming the previously described impact of pUL97 on pUL50 phosphorylation [8]. Notably, single pUL50 expression combined with lambda PP treatment also resulted in a partial mobility shift, even in the absence of viral pUL97 (Fig. 1a, upper panel; compare lanes 1 and 2). To further analyse the impact of cellular kinases, we performed phosphate affinity (Phos-tag) SDS-PAGE (Fig. 1b).Transient expression of full-length pUL50 did not provide a detectable impact of cellular kinase activity using this approach (data not shown). Therefore we transfected constructs for truncated versions of pUL50 and detected phosphorylated forms referring to pUL97 activity (Fig. 1b, lanes 2 and 5, asterisks). When catalytically inactive pUL97 (K355M) was coexpressed, this modification was not

Table 1. Importance of viral and cellular protein kinases for herpesviral nuclear egress

<table>
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<th>Kinase</th>
<th>Substrates relevant for nuclear egress</th>
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<tr>
<td>Alpha</td>
<td>HSV-1</td>
<td>pUS3</td>
<td>Lamin A/C, emerin, pUL34, pUL31</td>
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<td></td>
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<td>HSV-2</td>
<td>pUL13</td>
<td>Emerin, lamin B1</td>
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<td>HCMV</td>
<td>pUL97</td>
<td>Lamin A/C, B1</td>
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<td></td>
<td>MCMV</td>
<td>pUL97</td>
<td>Lamin A/C, pUL50, pUL53</td>
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<tr>
<td>Gamma</td>
<td>EBV</td>
<td>BGLF4</td>
<td>Lamin A/C</td>
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detectable (lanes 1 and 4). However, independent from pUL97 kinase activity, an additional form of pUL50 was observed, probably referring to endogenous cellular kinases (lanes 1, 2, 4 and 5, rhombuses). Strikingly, treatment with lambda PP could prevent the formation of these multiple phosphorylated pUL50 forms originating from viral and cellular kinase activity (Fig. 1b; compare lane 2 with lane 3, and lane 5 with lane 6). Further truncation of pUL50 abolished all of the modifications detected in the context of viral or cellular kinases (lanes 7 to 9).

As far as pUL53 phosphorylation is concerned, a similar impact of pUL97 has been described [8]. To additionally address the relevance of cellular kinases, 293T cells were used for the transient expression of pUL53-Flag alone or coexpression with pUL50-HA (Fig. 1c). Importantly, pUL53-Flag migrated faster on SDS-PAGE after incubation with lambda PP (Fig. 1c, lane 2), suggesting that pUL53 phosphorylation was abolished. In fact, this experiment showed that pUL53 was phosphorylated, even though viral pUL97 was not coexpressed. Notably, coexpression of pUL50 led to a more complex pattern of pUL53 phosphorylation, as indicated by lambda PP sensitivity (Fig. 1c; compare lanes 3 with 4), indicating that pUL50–pUL53 interaction promotes pUL53 phosphorylation. Taken together, our results indicate that both pUL50 and pUL53 can serve as substrates, not only for viral, but also for cellular protein kinases.

**pUL50 is phosphorylated at multiple sites during HCMV infection**

Due to the fact that pUL50 appears to be modified by a dual mode of pUL97-dependent and -independent phosphorylation, we performed phospho-specific mass spectrometry using pUL50 derived from HCMV-infected cells. HFFs were infected with HCMV strain AD169 or recombinant AD169-GFP UL50-HA, and pUL50 was immunoprecipitated from total lysates using a pUL50- or HA-specific antibody, respectively, or a combination of both. The immunoprecipitates were separated by SDS-PAGE and the isolated pUL50 bands (Fig. S1, available in the online Supplementary Material) were utilized for phospho-peptide enrichment in two independent laboratories (Fig. 2a). The phosphosites detected in both laboratories are highlighted in light grey. According to previous published data, we were able to confirm serine at position 216 (Ser216) as one of the dominant phosphorylation sites [8] and, in addition, a variety of phosphorylated residues within pUL50 were identified in this study. Two of these sites were consistently and safely detected by both platforms and were considered to be major sites, namely Ser300 and Ser324, which are located in central or C-terminal parts of the protein (Fig. 2b). Two more sites in the central domain, Ser300 and Ser324, which are located in central or C-terminal parts of the protein (Fig. 2b). Two more sites in the central domain, Ser300 and Ser324, which are located in central or C-terminal parts of the protein (Fig. 2b). Two more sites in the central domain, Ser300 and Ser324, which are located in central or C-terminal parts of the protein (Fig. 2b). Two more sites in the central domain, Ser300 and Ser324, which are located in central or C-terminal parts of the protein (Fig. 2b).
Western blot analyses. Inhibitors were added at the time point of 16 h and were refreshed 2 h prior the analyses of plasmid-transfected 293T or HeLa cells. Notably, only R25/ alsterpaullone (CDK1, 2 and 5 inhibitor) changed the nuclear rim localization of both pUL50 and pUL53 and induced a partial dyslocalization of both proteins to nuclear aggregates (Table 2, top line). The expression levels or general stability of both proteins did not change (data not shown). Moreover, the localization patterns of pUL50 and pUL53 remained unaltered under treatment with various other inhibitors of cellular kinases (Table 2). In order to further investigate the subcellular localization patterns of the core NEC during viral replication, HCMV-infected HFFs were likewise treated with kinase inhibitors. Also under these conditions, a R25-induced change in the pUL50–pUL53 colocalization to nuclear aggregates was confirmed as described recently (Fig. S2, panels e to h [16]). In contrast to this, other inhibitors did not induce any dyslocalization of the core NEC proteins (Fig. S2i–q). Notably, R25-mediated aggregate formation is considered to be an effect resulting from the inhibition of CKD activity, but not pUL97 activity. By performing additional Western blot experiments, we could exclude the possibility that R25 affects pUL97 (Fig. S3). However, R25 has also been reported as an effective inhibitor of glycogen synthase kinase 3 (GSK3). In order to address the question of whether GSK3 may be indirectly involved in core NEC formation, we transfected HeLa cells with plasmids coding for pUL50-HA and pUL53-Flag, and treated the cells with the GSK3 inhibitors lithium chloride (LiCl) or SB216763 (or DMSO as a control). After immunofluorescence staining of core NEC, confocal microscopy analysis revealed that only 10% (LiCl) or 15% (SB216763) of GSK3 inhibitor-treated cells showed some impairment of pUL50–pUL53 rim formation (5% DMSO; data not shown), which is clearly distinguishable from R25, for which our previous quantitative determination showed a predominant and clearly detectable loss of rim formation in 61.9% of the cells [16]. Taken together, CKD activity specifically, but so far no other detectable cellular kinase activity, appears to be important for efficient rim colocalization between pUL50 and pUL53 (with a particular focus on the R25-targeted CDKs 1, 2 or 5).

**pUL50 is a substrate of *in vitro* phosphorylation by the cellular kinases CDK1 and PKCα**

Our recently published results showing that both cellular kinases, PKCα and CDK1, interact directly with core NEC protein pUL50 [16, 18, 19] pointed to the possibility that pUL50 may also be phosphorylated as one of their substrates. To analyse the *in vitro* phosphorylation of pUL50, catalytically active or inactive versions of CDK1 and PKCα were transiently coexpressed with pUL50 in 293T cells and immunoprecipitated using tag- or protein-specific antibodies. Then, lysates were subjected to kinase-specific IVKA reactions to investigate phosphorylation (Fig. 3). As a clear confirmation of our earlier results, the phosphorylation of pUL50-HA by CDK1-HA (Fig. 3a, lanes 2–3) or PKCα-Flag (Fig. 3b, lane 2) was detectable. No pUL50 phosphorylation...
signal was seen when empty vector (Fig. 3a, lane 1; Fig. 3b, lane 2) or catalytically inactive PKCa(K368D)-HA (Fig. 3b, lane 3) were used. In both experiments, the staining of lysates and IP samples taken prior to IVKA analyses demonstrated comparable levels of transiently expressed proteins (Fig. 3a and b; indicated by lysates or IP control). As specificity controls, we obtained strong signals for the phosphorylation of histones (Fig. 3a, lane 2; Fig. 3b, lane 1), cyclin B1 phosphorylation by CDK1 (Fig. 3a, lane 4) and PKCa–Flag autophosphorylation (Fig. 3b, lanes 1–2). Thus, *in vitro* phosphorylation of pUL50 by CDK1 and PKCa could be demonstrated, indicating a possible role for these protein kinases in NEC regulation.

**pUL53 is also phosphorylated in a CDK1-dependent manner**

To analyse the putative phosphorylation of pUL53 by CDK1, an additional IVKA was performed under the conditions described above. A moderate level of CDK1-mediated phosphorylation of pUL53 could be detected, which was abolished under R25 treatment (Fig. 4a; compare lanes 2 and 3). We next examined the putative CoIP-detectable binding of pUL53 to CDK1 (Fig. 4b) or CDK2 (Fig. S6). Intriguingly, no signal for direct interaction was detectable (Fig. 4b, Fig. S6, lane 3). In light of the slightly positive result for CDK1-mediated phosphorylation of pUL53, the negative CoIP result may be due to a weak-affinity type of substrate–kinase interaction, possibly below the CoIP detection limit. Concerning the control settings, CoIPs were considered to be specific on the basis of detectable CDK–cyclin interactions (Figs 4b and S6, lane 1). Thus, although direct pUL53–CDK1 interaction was not detectable, it is very likely that CDK1 (mainly recruited through pUL50 interaction) may also contribute to pUL53 phosphorylation, and possibly the phosphorylation of further NEC-associated proteins.

**Assessment of PKCa knock-out cells regarding functionality of the HCMV core NEC**

Although PKCa has been identified as a cellular kinase that is involved in the phosphorylation of egress-relevant proteins of human and murine cytomegaloviruses (data from this study and [14, 16, 18]), no functional importance for the regulation of viral nuclear egress has been revealed so far. To address this issue, we generated PKCa knock-out cells using the CRISPR/Cas9 system [20, 21]. In order to verify the efficacy of knock-out, we lysed HFFs after the transduction with lentiviral transfer constructs to analyse the expression of classical PKCa and PKCy, as well as novel PKCa*. As an initial finding, we noted that PKCa knock-out was achieved at levels of differential efficacy, i.e. one of the gRNAs, similar to empty vector, did not alter PKCa levels (Fig. S4, lane 1 and 2), whereas a partial to complete depletion of PKCa was obtained with other gRNAs (Fig. S4, lanes 3–4, upper panel). These results between PKCa knock-out cells could result from the variability provided by the non-clonal nature of the selected cell populations or that provided by a homozygous double knock-out versus a heterozygous single-allele knock-out. Since the members of the PK family share functional overlaps [22], we also investigated the expression of two PKC isoforms that are not directly subject to the gRNA targeting strategy. Interestingly, neither the PKCy nor the PKCa protein levels were altered, thereby excluding gRNA mistargeting effects. Next, we investigated viral protein expression by using total lysates for HCMV-infected PKCa knock-out cells, PKCa (KO), stained for viral immediate early (IE), early (E) and late (L) proteins. PKCa knock-out was stable throughout the course of infection (Fig. S5a, upper panel), but no impact on IE, E or L protein expression was observed after 4 days post-infection (Fig. S5a). For the further characterization of PKCa(KO) cells, we infected HFFs at a low multiplicity of infection (m.o.i.) of 0.01 and used sensitive quantitative real-time PCR to quantitate IE1 genome equivalents in culture supernatants (Fig. S5b). In all cell populations, HCMV showed a wild-type-like replication efficiency compared to controls, thereby excluding a general effect on HCMV replication (Fig. S5c). Thus, PKCa does apparently interact with the HCMV core NEC and plays a role in NEC protein phosphorylation, but functional consequences could not be identified under the described conditions, so that its regulatory impact on HCMV nuclear egress remains in question.

**Table 2. Influence of cellular kinase inhibitors on core NEC assembly and stability**

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<th>Inhibitor</th>
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Assessment of CDK1 knock-down or CDK inhibition with regard to the functionality of the HCMV core NEC

Due to the fact that CDK1 is an essential protein in cell cycle control and complete CDK1 knock-out appears to be difficult, we used a CDK1 knock-down approach to address its functional importance for the NEC. HFFs were transfected with CDK1-specific siRNAs and subsequently used for infection with HCMV AD169 (1 day post-transfection; Fig. 5). Protein samples were taken at the time points indicated, showing that efficient CDK1 knock-down was achieved in transient periods until 2 d p.i. Starting with 3 d p.i., however, CDK1 levels began to normalize, as shown for siRNA s463 (Fig. 5, lane 11). Furthermore, as an interesting finding, we noted that HCMV IE1 expression was moderately decreased, an effect which also reversed at 4 d p.i. For early or early/late proteins (pUL50, pUL53 and pUL97), expression was slightly below that for the control cells. Of note, CDK1 knock-down did not affect the expression of the true late protein MCP. Thus, the knock-down of CDK1 affected viral protein expression to a limited extent.

In the next step, the known antiviral activity of the CDK inhibitor R25/alsterpaullone was used as a tool to study the relevance of CDK1 for the functionality of the HCMV NEC. First, the HCMV GFP-based replication assay was used to quantitate the anti-HCMV efficacy of R25. HCMV replication was inhibited by R25 in a concentration-dependent manner with a 50% effective concentration (EC50) of 4.72 ±0.23 µM (Fig. 6a). No signs of cytotoxicity including the growth behaviour and morphology of the primary HFF cultures, were detected by microscopic inspection within this range of concentrations (data not shown). Next, we addressed the question of whether CDK1 activity is required for pUL50 phosphorylation. Immunoprecipitated proteins derived from...
lysates of cotransfected 293T cells were used for IVKA analyses performed under R25 treatment (Fig. 6b). As a clear finding, pUL50, which was strongly phosphorylated by the coimmunoprecipitated CDK1-HA in the absence of R25, remained unphosphorylated in the presence of R25 (Fig. 6b; compare lanes 3 and 4). Weak pUL50 phosphorylation was also detected when CDK1-HA was not coexpressed, indicating that endogenous CDK1, also present in the precipitates (Fig. 6b, middle panel of IP controls), is sufficient for pUL50 phosphorylation (Fig. 6b, lane 2). Staining of the control blots indicated that comparable amounts of pUL50 and CDK1 were subjected to IVKA reactions (Fig. 6b, IP control and lysates).

In the final step, we analysed the possible impact of R25 on pUL50–pUL53 interaction. For this purpose, pUL50-HA and pUL53-Flag were assayed in a CoIP setting under R25 treatment (R25 had already been added during cell lysis in order to suppress CDK activity throughout the period of experimentation). The specificity of the CoIP reactions was assured by controls with DMSO (Fig. 6c, lanes 2–3). Notably, R25 partially reduced the signals of CoIP interaction for pUL53 or the expression levels of some pUL53 varieties (Fig. 6c; see the different phosphorylated forms marked by the parentheses), but did not completely block pUL50 interaction (Fig. 6c, upper panel, lane 4). Hence, R25 is not capable of fully preventing pUL50–pUL53 interaction under these conditions, but appears to interfere with the interaction capacity of specific phosphorylated forms of pUL53. These findings underline our conclusion that CDK1 (possibly involving further CDKs) has a regulatory impact on the phosphorylation and assembly of the HCMV core NEC and on HCMV replication.

**DISCUSSION**

Although core NEC phosphorylation by viral-encoded kinases has been shown, the impact of cellular kinases on core NEC function remains enigmatic. For HCMV it has been proposed that the phosphorylation of core NEC is mediated solely by pUL97, thereby promoting proper core NEC formation [8]. This finding was challenged by identification of the fact that cellular kinases, namely CDKs, also influence the localization of the HCMV core NEC [16]. In this study, we further analysed the impact of endogenous kinases for HCMV-specific NEC. The main findings are: (i) the core NEC is not solely phosphorylated by viral pUL97; (ii) cellular kinases CDK1 and...
PKCα can phosphorylate pUL50 in vitro; (iii) the impact of PKCα phosphorylation on NEC functionality has not been identified; and (iv) CDK1-dependent pUL50 phosphorylation regulates core NEC interaction, nuclear rim colocalization and the efficiency of viral protein production.

Using mass spectrometry approaches, pUL50 phosphorylation by pUL97 has been demonstrated to occur at Ser216 [8]. Our novel data confirm this finding on the basis of Western blot and Phos-tag analyses using proteins from coexpression settings of pUL50 and pUL97. We were able to...
prevent pUL50 phosphorylation by pUL97 via lambda PP experiments, with this being visible from the loss of modified bands or increased PAGE motility. Phos-tag analysis revealed that the pUL50 expression pattern can include specific protein varieties. For the hyperphosphorylation of pUL50, viral kinase activity is required, whereas in the absence of viral kinase activity, a different band or protein variety was detected, most likely resulting from endogenous cellular kinase activity. Notably, deletion of amino acids 251 to 280 abolished the formation of viral and cellular kinase-dependent forms of pUL50. Two putative phosphorylation sites are located in this protein region, i.e. Ser251 and Ser280. Based on our mass spectrometric analysis, these residues were defined as minor phosphosites that might be phosphorylated independently of the viral kinase pUL97 in HCMV-infected cells.

The general importance of cellular kinases for efficient HCMV replication has been suggested previously [23]. Here, we provide the first evidence that pUL50 is phosphorylated by both CDK1 and PKCa in vitro, with possible functional consequences in the case of CDK1-mediated phosphorylation. Members of the PKC superfamily have been suggested to participate in herpesviral nuclear egress [11, 12, 14]. For HSV-1, it has been postulated that PKCδ is specifically required for emerin and lamin B phosphorylation, although the inhibition of PKCδ does not inhibit viral growth. Furthermore, experiments with broad PKC inhibitors reduced viral replication up to fivefold and also prevented efficient nuclear capsid egress [12]. Moreover, PKCa interaction with the HCMV NEC has been demonstrated repeatedly, although, in line with previous observations, PKCa may be located primarily in cytoplasmic virion assembly complex (cVAC) and it may possess no or only minor functional importance for nuclear egress [16, 18, 19, 24]. According to our results, isoform-specific PKCa knock-out had no detectable effect on HCMV replication or core NEC formation. It should be considered, however, that PKCa may be functionally replaced by pUL97 or other PKC isoforms. Due to their high structural similarity, PKCs share a variety of mutual functions that can be taken over by other members of the PKC family [22]. On the other hand, CDKs are well-known to play a crucial role during HCMV replication [23, 25]. The broad inhibition of CDKs has been shown to block HCMV replication [26], and thus our finding that the CDK1, 2 and 5 inhibitor R25 impairs viral growth in a dose-dependent manner was not unexpected. Intriguingly, the phosphorylation of both core NEC proteins is dependent on CDK1 activity. Blocking activity by R25 led to a loss of pUL50 or pUL53 phosphorylation, which might be a prerequisite of both proteins for proper core NEC formation. In addition to a block of NEC phosphorylation, R25 may induce the dyslocalization of intranuclear pUL50–pUL53, although we found that nuclear rim recruitment and distinct pUL50–pUL53 colocalization is not completely lost under R25 treatment. In line with this, our CoIP analysis revealed that blocking CDK phosphorylation prevented the NEC interaction capacity of specific forms of phosphorylated pUL53, but did not produce a complete block for interaction. Of note, knock-down of CDK1 resulted in moderate inhibitory effects on viral protein expression. Therefore, stable knock-down for a longer period might be beneficial for drawing further conclusions regarding CDK1-dependent effects on viral protein levels.

We utilized mass spectrometry for the identification of phosphorylated residues within pUL50 and classified the identified scores as major and minor pUL50 phosphosites. However, we still need to determine the circumstances in which viral or cellular or both types of protein kinases are responsible for phosphorylation in detail for each of these sites. In summary, our study provides novel insights into the involvement of cellular protein kinases in HCMV-specific core NEC regulation. Importantly, CDK-mediated phosphorylation appears to coregulate the formation of the core NEC as a rate-limiting step for nuclear egress and the efficiency of HCMV replication.

METHODS

Plasmid constructs

The expression plasmids coding for the following HA- or Flag-tagged versions of HCMV and cellular proteins were described previously: HCMV proteins pUL53-Flag, pUL50-HA, pUL50(1-310)-HA, pUL50(1-280)-HA, pUL50(1-250)-HA, pUL97-Flag, pUL97(K355M)-Flag, pCMV-CDK1-HA, pCMV-CDK2-HA [27], PKCa(K368D)-HA (kindly provided by Huang Shuang, Georgia, USA) and PKCa-Flag [18, 19]. As a vector control, pcDNA3.1 (Invitrogen) was used in coimmunoprecipitation assays or in vitro kinase assays.

Cell culture, plasmid or siRNA transfection, phosphate affinity (Phos-tag) SDS-PAGE and lambda phosphatase (lambda PP) treatment

Human embryonic epithelial 293T cells (ATCC CRL-3216) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal calf serum (FCS) and 100 mg gentamicin ml⁻¹. Primary human foreskin fibroblasts (HFF) were cultivated in minimum essential medium (MEM) containing 7.5 or 10% FCS and 100 mg gentamicin ml⁻¹. HFFs transduced with lentiviral transfer constructs were additionally cultivated with 5 µg puromycin (Serva) ml⁻¹. Transient plasmid transfection of 293T cells was performed using polyethyleneimine–DNA complexes (Sigma-Aldrich) as described previously [28]. For transient knock-down of CDK1, HFF cells were transfected with 60 pmol siRNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Synthetic siRNAs specific for CDK1 (S463, cat. #4392420 and s464, cat. #4390824) were obtained from Thermo Fisher Scientific (Waltham, USA), with Silencer negative control no. 1 siRNA (AM4611) being used as a specificity control. For the identification of phosphorylated protein varieties, Phos-tag reagent (AAL-107) [29] was purchased from Wako Pure Chemical Industries and used according to the
manufacturer’s instructions. For experiments with lambda phosphatase, cells were first lysed in CoIP buffer [16] and then proteins were precipitated via tag-specific antibodies as described previously [16]. After immunoprecipitation, proteins were suspended in CoIP buffer without EDTA and supplemented with 1 × PMP buffer, 1 mM MnCl₂ and 0.5 µl of lambda PP (provided from New England Biolabs, P0753S). After incubation at 30 °C for 30 min, samples were denatured at 95 °C for 10 min and analysed by standard Western blot procedures.

**Commmunoprecipitation (CoIP) assay and in vitro kinase assay (IVKA)**

For both CoIP and IVKA, 293T cells were seeded into 10 cm dishes with a density of 5.0 × 10⁶ cells. Two to three days post-transfection, CoIP [16] or IVKA [18] was performed as described previously. Immunoprecipitation control samples of approximately one-sixth of the total volumes were taken prior to IVKA reactions.

**HCMV infection, GFP-based HCMV replication assay, multistep growth curve analysis**

HCMV infection experiments were performed at an m.o.i. of 1.0 (or higher as indicated for specific experiments) using HCMV strains AD169, AD169-GFP [30] or AD169-GFP UL50-HA [31]. After incubation for 90–120 min at 37 °C, virus inoculi were removed and replaced with fresh growth medium. For mass spectrometry analysis, confluent cell culture flasks with HFFs were infected with AD169-GFP UL50-HA. Four to five days post-infection, the cells were harvested and lysed in CoIP buffer. GFP-based replication assay was performed as described previously [30, 32]. Multi-step growth curve analyses of infected HFF cells were performed as described in a previous study [33].

**Antibodies**

The following monoclonal (mAb) and polyclonal (pAb) antibodies were used: mouse pAb-HA (T501, Signal Antibody), mouse mAb-HA (clone HA-7, Sigma-Aldrich), mouse mAb-Flag (1804, Sigma-Aldrich), mouse pAb-UL53 (kindly provided by Paola Dal Monte, Bologna, Italy), mouse mAb-UL50 (UL50.01), mouse mAb-UL53 (UL53.01), mouse mAb-UL97 (1C4/0.2; kindly provided by Stipan Jonjic and Tihana Lenac, University of Rijeka, Croatia), mouse mAb-UL44 (kindly provided by Bodo Plachter, University of Mainz, Germany), mouse mAb-IE1p72 and mAb-MCP (major capsid protein; kindly provided by William Britt, University of Alabama, USA), mouse mAb-β-actin (AC-15, Sigma-Aldrich), rabbit pAb-cyclin B1 (sc-752, Santa Cruz), rabbit pAb-cdc2 p34 (CDK1, sc-954, Santa Cruz), rabbit pAb-CDK2 (sc-163, Santa Cruz), rabbit pAb-PKCα (sc-211, Santa Cruz) and rabbit pAb-PKCβ (sc-937, Santa Cruz). The secondary antibodies were Alexa Fluor 488-/555-conjugated secondary antibodies for indirect immunofluorescence (Molecular Probes) and horseradish peroxidase-conjugated anti-mouse/-rabbit secondary antibodies for Western blot analyses (Dianova).

**Protein kinase inhibitors**

The drugs used in the present study were obtained from various sources: the CDK inhibitors R25 (also known as alsterpaullone) [34] and R22 [35] were provided by GPC Biotech AG ( Martinsried, Germany) and the CDK7-specific inhibitor LDC4297 [32] was provided by Lead Discovery Centre GmbH (Dortmund, Germany). The PKC-specific inhibitors rottlerin (ACC Corporation; specifically inhibits PKC isofrom δ), Gö6976 (Sigma-Aldrich; inhibition of PKCa, β1 and, additionally, the viral kinase pUL97), Gö6784 (Calbiochem; broad inhibitor of PKC isoforms), the SK3 inhibitors lithium chloride (LiCl) (Merck, Darmstadt, Germany) and SB216763 (Sigma-Aldrich, Taufkirchen, Germany), as well as the pUL97-specific maribavir (MBV) [36, 37] (synthesized by Shanghai PI Chemicals Ltd, People’s Republic of China) were obtained from commercial sources. The compounds were dissolved in dimethylsulfoxide (DMSO) and stored at −20 °C according to the manufacturers’ instructions.

**Indirect immunofluorescence assay and confocal laser-scanning microscopy**

HFFs were grown on coverslips for infection experiments. At 4 days p.i., the cells were fixed with 4 % paraformaldehyde solution and subsequently permeabilized by incubation with 0.2 % Triton X-100 solution. Non-specific antibody staining was prevented by a blocking incubation step with 2 mg ml⁻¹ human γ-globulin (Cohn fraction II, Sigma Aldrich) followed by indirect immunofluorescence staining as described previously [18, 19]. The coverslips were analysed using a TCS SP5 confocal laser-scanning microscope (Leica). For three-dimensional images, z-series were recorded along 12 µm (z-axis) with a pinhole of 1 Airy unit. Images and z-series were analysed using LAS AF software (Leica Microsystems).

**Phosphosite identification by nanoLC-MS/MS analysis**

**Procedures applied by proteomics laboratory EDyP Grenoble**

Proteins were prepared and in-gel digested as previously described [3]. Phosphopeptides were enriched from the resulting peptides using titanium dioxide beads (TitanSphere, GL Sciences, Inc.) and adapting a protocol described by others [38]. Briefly, peptides were solubilized in 80 % AcN, 5 % TFA and 1 M glycolic acid (loading buffer) and incubated in the batch mode with 0.6 mg ml⁻¹ of TiO₂ beads for 1 h under shaking. After centrifugation, the pellet was washed with loading buffer, then washing buffer 1 (80 % AcN, 1 % TFA), and then washing buffer 2 (10 % AcN, 0.1 TFA) before the elution of phosphopeptides using in 1.12 % ammonia in H₂O. The eluted peptides were then acidified before nanoLC-MS/MS analyses were performed using the Ultimate 3000 RSLCnano and the Q-Exactive Plus (Thermo Fisher Scientific). Peptides were sampled on a 300 µm × 5 mm PepMap C18 precolumn and separated on a 75 µm × 250-mm C18 column (PepMap, Dionex). The nano-LC method consisted of a 40 min gradient at a flow rate of 300 nL min⁻¹, ranging from
The peptides were extracted from gel bands as described previously [40, 41]. Extracted peptides were desalted on C18 stage tips prior to LC-MS/MS analysis and were vacuum-dried to resuspend in 0.1% trifluoroacetic acid (TFA). All processes were performed on ice and the peptides were analysed promptly after extraction. A 2 µl aliquot from each sample was analysed in triplicate by an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were loaded and separated on (300 µm i.d. × 5 mm) PepMap100 columns using the UltiMate 3000 RSLC nano UHPLC system ( Dionex, Sunnyvale, CA, USA) with a 3–35% gradient of acetonitrile in 0.1% formic acid for 165 min at a flow rate of 300 nL min⁻¹ at 35 °C. The eluted peptides were ionized with nanospray Flex ion source (Thermo Fisher Scientific) and measured with a survey scan range of 300–2000 m/z. The most intense peptides from each scan cycle were selected and fragmented using collision-induced dissociation (normalized collision energy 35%, activation time 250 ms and isolation width 1.6 m/z), and the resulting MS/MS scans were analysed in an ion trap. Dynamic exclusion was enabled for 60 s and excluded after one use. The raw data files were processed against Homo sapiens proteome as comprised in Uniprot (status: 18/02/16, containing 145 837 sequences) using Proteome Discoverer (version 1.14, Thermo Fisher Scientific). HCMV sequences of pUL50, pUL53, and pUL80 were searched against the measured spectra with the following parameters: carbamidomethyl (C, fixed), acetyl (Protein N-ter, variable), oxidation (M, variable) Phospho (ST, variable) and Phospho (Y, variable). Proline software (http://proline.profiproteomics.fr) was used to filter the results (conservation of rank 1 peptides; peptide length ≥7; minimum PSM score of 25; P<0.01 for query homology threshold; minimum of one specific peptide per identified protein group). Only phosphopeptides exhibiting a modification probability ≥80% (based on MD score [39]) were further considered.

Procedures applied by proteomics laboratory FAU Erlangen

Peptides were extracted from gel bands as described previously [40, 41]. Extracted peptides were desalted on C18 stage tips prior to LC-MS/MS analysis and were vacuum-dried to resuspend in 0.1% trifluoroacetic acid (TFA). All processes were performed on ice and the peptides were analysed promptly after extraction. A 2 µl aliquot from each sample was analysed in triplicate by an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were loaded and separated on (300 µm i.d. × 5 mm) PepMap100 columns using the UltiMate 3000 RSLC nano UHPLC system (Dionex, Sunnyvale, CA, USA) with a 3–35% gradient of acetonitrile in 0.1% formic acid for 165 min at a flow rate of 300 nL min⁻¹ at 35 °C. The eluted peptides were ionized with nanospray Flex ion source (Thermo Fisher Scientific) and measured with a survey scan range of 300–2000 m/z. The most intense peptides from each scan cycle were selected and fragmented using collision-induced dissociation (normalized collision energy 35%, activation time 250 ms and isolation width 1.6 m/z), and the resulting MS/MS scans were analysed in an ion trap. Dynamic exclusion was enabled for 60 s and excluded after one use. The raw data files were processed against Homo sapiens proteome as comprised in Uniprot (status: 18/02/16, containing 145 837 sequences) using Proteome Discoverer (version 1.14, Thermo Fisher Scientific). HCMV sequences of pUL50, pUL53, and pUL80 were searched against the measured spectra with the following parameters: carbamidomethyl modification was set as fixed and the oxidation of methionine and phosphorylation of serine, threonine and tyrosine residues was set as an optional modification. The mass tolerance was set to 10 p.p.m. for survey scans and 0.6 Da for fragment mass measurements. Peptide charges of 2–7 were allowed. Only the resulting peptides with a false discovery rate (FDR) below 1% were regarded as identified [40, 41]. The raw files were simultaneously evaluated by Phospho RS (Thermo Fisher Scientific) and Peaks Q (Bioinformatics Solution, Inc., Canada) software for highly confident phosphosite identification (≥80% probability) at serine, threonine and/or tyrosine residues.

Design of plasmids harbouring PKCα-specific genomic RNAs (gRNAs)

For the design of gRNAs specific for PKCα (NCBI NG_012206.1), the CRISPR design tool from Zhang Laboratories (MIT 2015, http://crispr.mit.edu:8079/) was used. Three different gRNAs were chosen targeting sequences within exon 1 or 3 with a score of 95. The generation of plasmids containing these PKCα-specific gRNAs was performed according to the standard protocol (https://www.addgene.org/static/cms/files/Zhang_lab_LentiCRISPR_library_protocol.pdf) by cloning gRNAs into CRISPR vector pLentiCRISPRv2. The sequences of oligonucleotides for gRNA hybridization are given in Table S1.

Generation of lentiviral transfer constructs and transduction of HFFs

In order to generate replication-deficient lentiviruses, 293T cells were cotransfected with a pLentiCRISPRv2 vector coding for one of the three different gRNAs targeting genomic PKCα, together with a packaging plasmid mix coding for HIV-1 Gag/Pol, HIV-1 Rev and the envelope protein G of vesicular stomatitis virus (expression vectors pLP1, pLP2 and pVSV-G, respectively) using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). Control supernatants were derived from transfection with empty vector pLentiCRISPRv2. At 48 h post-transfection, lentiviral supernatants were subjected to centrifugation in order to remove debris, filtered and stored in aliquots at –80 °C. HFF cells of a low passage number were incubated for 24 h with various amounts of lentiviral supernatants in the presence of 7.5 µg ml⁻¹ polybrene (Sigma-Aldrich, Taufkirchen, Germany). Next, the virus supernatants were replaced by fresh media and the cells were incubated for another 24 h before 5 µg puromycin ml⁻¹ was added, resulting in the selection of stably transduced cell populations.

Funding information

This work was supported by Deutsche Forschungsgemeinschaft (SFB796 subprojects C3, C2 and Z; MA1289/8-1; MI2143/2-1) and DAAD-Go8 (grant MM/JM/WDR/SH 2017-18). The proteomic experiments were partially supported by a ProFi grant (ANR-10-INBS-08-01).

Acknowledgements

The authors are grateful to all members of the M.M. research laboratory, in particular Mirjam Steinrueger and Corina Hutterer for their contributions in the analysis of viral and cellular protein kinases. We also acknowledge support from Anna Reichel, who provided information about GSK3 inhibitors, as well as highly valuable cooperation from Thomas Stamminger and his team. We would like to thank the discovery platform and informatics group at EdYp Grenoble for additional support.
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