New insight into the phosphorylation-regulated intranuclear localization of human cytomegalovirus pUL69 mediated by cyclin-dependent kinases (CDKs) and viral CDK orthologue pUL97

Laura Graf,1† Sabine Feichtinger,1 Zin Naing,2 Corina Hutterer,1 Jens Milbradt,1 Rike Webel,1 Sabrina Wagner,1 Gillian M. Scott,2 Stuart T. Hamilton,2 William D. Rawlinson,2 Thomas Stamminger,1 Marco Thomas1 and Manfred Marschall1

Correspondence
Manfred Marschall
manfred.marschall@viro.med.uni-erlangen.de

1Institute for Clinical and Molecular Virology, Friedrich-Alexander University of Erlangen-Nürnberg, Erlangen, Germany
2Virology Division, SEALS Microbiology, Prince of Wales Hospital, University of New South Wales, Sydney, Australia

Cyclin-dependent kinases (CDKs) are multifaceted regulators involved in the replication of human cytomegalovirus. Recently, we demonstrated an interaction of CDK9–cyclin T1 as well as viral CDK orthologue pUL97 with the viral regulator pUL69, thereby leading to pUL69-activating phosphorylation. Here, we demonstrate that colocalization and direct pUL69–cyclin T1 interaction is independent of viral strains and host cell types. In vitro phosphorylation of pUL69 by CDK9 or pUL97 did not occur in a single site-specific manner, but at multiple sites. The previously described fine-speckled nuclear aggregation of pUL69 was assigned to the late phase of viral replication. CDK inhibitors, including a novel inhibitor of the CDK-activating kinase CDK7, massively intensified this fine-speckled accumulation. Interestingly, we also observed spontaneous pUL69 accumulation in the absence of inhibitors at a lower frequency. These findings provide new insight into pUL69 kinase interregulation and emphasize the importance of pUL69 phosphorylation for correct intranuclear localization.

Human cytomegalovirus (HCMV; Human herpesvirus 5; family Herpesviridae) is a human pathogen distributed worldwide. Although primary HCMV infection of the immunocompetent host typically remains asymptomatic, severe disease can occur upon infection of immunonaive and immunocompromised individuals, such as neonates, transplant recipients and cancer or AIDS patients (Mocarski et al., 2013). HCMV pathogenicity and replication efficiency are significantly coregulated by a close functional linkage between viral and host cell processes (Marschall et al., 2011). HCMV infection modulates the expression and activity of cyclin-dependent protein kinases (CDKs) and requires CDK activity, in particular CDK1, 2, 7 and 9, at various stages, such as regulation of viral gene expression, protein phosphorylation and intracellular localization (Jault et al., 1995; Fortunato et al., 2000; Sanchez et al., 2004; Schang, 2004; Tamrakar et al., 2005; Sanchez & Spector, 2006; Bain & Sinclair, 2007; Hertel et al., 2007; Kapasi & Spector, 2008; Kapasi et al., 2009; Rechter et al., 2009; Feichtinger et al., 2011; Marschall et al., 2011; Milbradt et al., 2014; Hutterer et al., 2015).

CDKs are heterodimeric serine/threonine kinases that become activated upon binding to cyclins. As they are involved in the regulation of multiple cellular processes, CDKs are subdivided into major functional groups, i.e. cell cycle-associated CDKs (primarily CDK1, 2, 4, 6 and 7), transcription-regulating CDKs (primarily CDK7, 8 and 9) and CDKs with other specialized functions (Bregman et al., 2000). Interestingly, CDKs are also functionally integrated into the replication of herpesviruses. Notably, the HCMV-encoded protein kinase pUL97 shares important structural and functional features with CDKs, such as structural similarities in the N- and C-terminal lobe of the kinase domain, interaction with cyclins, phosphorylation of identical substrates, and functional complementation in heterologous systems (Chou et al., 2006; Romaker et al., 2006; Chou, 2008; Hume et al., 2008; Hamirally et al., 2009; Kamil et al., 2009; Thomas et al., 2009; Kuny et al., 2010; Graf et al., 2013; Iwahori...
et al., 2015; Oberstein et al., 2015; Steingruber et al., 2015). A combined regulatory impact of CDK and pUL97 activity on the viral mRNA export factor pUL69 was demonstrated (Rechter et al., 2009; Thomas et al., 2009; Feichtinger et al., 2011; Oberstein et al., 2015) and, very recently, additional posttranslational modification by methylation proved to be similarly important (Thomas et al., 2015). A pronounced kinase inhibitor-induced formation of nuclear speckled aggregation of pUL69 was originally demonstrated by Sanchez & Spector (2006). In more recent reports, speckle formation was experimentally addressed by the use of broad CDK inhibitors (Rechter et al., 2009) or the selective CDK9 inhibitor R22 (Feichtinger et al., 2011) as well as a panel of pUL97 inhibitors (Thomas et al., 2009). Both CDK9 and viral kinase pUL97 proved to be involved in a phosphorylation-specific regulation of pUL69 mRNA transport activity (Thomas et al., 2009; Rechter et al., 2009; Feichtinger et al., 2011; Marshall et al., 2011). Interestingly, direct interactions between pUL69 and cyclin T1, as well as between pUL97 and several types of cyclins, have been demonstrated recently (Graf et al., 2013; Steingruber et al., 2015).

In the present study, we analysed determinants of the phosphorylation-regulated intranuclear localization of pUL69, specifically focusing on CDKs and viral pUL97. First, we investigated the nuclear localization of pUL69 in late-phase viral replication centres of HCMV-infected fibroblasts (Rechter et al., 2009). Human foreskin fibroblasts (HFFs), embryonic fibroblasts (MRC-5), retinal epithelial cells (ARPE-19) and placental trophoblasts (TEV-1) were cultivated as described previously (Marschall et al., 2000; Hamilton et al., 2014) and used for infection (m.o.i 0.1–0.5) with HCMV laboratory strain AD169 (varUK; Cunningham et al., 2010) or a BAcmid-derived recombinant of the WT-like strain Merlin (Stanton et al., 2010). In all settings analysed, nuclear accumulation of pUL69 was primarily detectable in viral nuclear replication centres (Fig. 1a, panels c, h, m, r; compare with data available in the online Supplementary Material). A partial colocalization in these distinct granular structures was observed for cyclin T1 (Fig. 1a, panels d, i, n, s and h, g, l, q), which was smoothly distributed in a nuclear or nucleo-cytoplasmic localization in mock-infected controls (Fig. 1a, panels a, f, k, p). Thus, intranuclear colocalization between pUL69 and cyclin T1 was consistently observed late during HCMV replication, and was not restricted to specific viral strains or host cell types.

In order to confirm that the interaction between pUL69 and cyclin T1 was independent of the virus strain, we addressed the question whether direct pUL69–cyclin T1 interaction was detectable by coimmunoprecipitation (CoIP) from infected-cell proteins using the WT-like HCMV strain Merlin (Stanton et al., 2010). For this purpose, HFFs were infected with HCMV Merlin for 6 days before total lysates were prepared to perform CoIP analysis under conditions described for a similar setting with laboratory strain AD169 (Feichtinger et al., 2011; Graf et al., 2013). An upregulation of cyclin T1 and CDK9 was observed in HCMV-infected cells (Fig. 1b, lanes 6–8; note that quantitative variation is explained by a limited stability of these protein complexes) compared with the mock-infected control (Fig. 1b, lane 5). Using a cyclin T1-specific polyclonal antibody, the CoIP of pUL69 and CDK9 was shown (Fig. 1b, lane 2). The quantity of CDK9 coimmunoprecipitated together with cyclin T1 (CoIP signal set as 100 %) was higher (135 %) compared with that of pUL69 (36 %). The pUL69–cyclin T1 interaction was considered specific as control reactions without antibody or with an irrelevant aldolase-specific antibody remained at marginal levels (4–3 %; Fig. 1b, lanes 3 and 4). This finding underlines the association of pUL69 with cyclin T1 in protein complexes regularly formed during infection with strains of HCMV in various host cell types.

Next, we analysed the patterns of in vitro phosphorylation of WT and mutants of pUL69 (i.e. amino acid replacement mutations in putative phosphorylation sites) that were exposed to the kinases CDK9 or pUL97 under established conditions of in vitro kinase assays (IVKAs) (Marschall et al., 2002; Thomas et al., 2009; Rechter et al., 2009; Webel et al., 2014). Replacement mutants were generated on the basis of pUL69-Flag cloned in vector pcDNA3.1 by the use of a protocol for site-directed mutagenesis described previously (Lischka et al., 2006; Thomas et al., 2009, 2015; Schmeiser et al., 2013) (see Table S1 for PCR primers and a description of constructs; Invitrogen/Life Technologies). For IVKAs, proteins were either transiently expressed in plasmid-transfected 293T cells and immunoprecipitated by the use of mAb-Flag [pUL69-Flag and pUL97(181–707)-Flag] (Thomas et al., 2009) or exogenously added to the reactions in the form of affinity-purified proteins (CDK9-cyclin T1, histone 2B and Rb-CTF; Proquinase) (Rechter et al., 2009). The reliability of transfection and immunoprecipitation efficiencies were monitored by analysing aliquots of the samples by Western blotting in parallel (data not shown). Proteins were analysed in pUL97- or CDK9-specific IVKA reactions to identify specific sites of pUL69 phosphorylation (Fig. 2). We demonstrated that both kinases phosphorylate pUL69, and that the efficiencies of in vitro phosphorylation were very similar for pUL97 and CDK9. Notably, mutations did not affect phosphorylation, i.e. neither of the replacements of putative serine/threonine target residues within the N-terminus of pUL69 abrogated phosphorylation (Fig. 2, lane 3, WT; lanes 5–11, replacement mutants). Negative control reactions showed only background signals (Fig. 2: lane 4, pUL69 without kinase; lane 2, kinase without pUL69). Positive control reactions (Fig. 2: lane 1, IVKA standard substrates histone 2B or Rb-CTF) verified the reliability of the assay. A quantification of signal intensities confirmed the even level of phosphorylation of pUL69 WT and mutants (Fig. S1). These findings provide novel evidence for the dual phosphorylation of pUL69 by both cellular and viral protein kinases. Notably, phosphorylation of
**Fig. 1.** Characterization of the interaction between pUL69 and cyclin T1 in HCMV-infected cells. (a) Intranuclear colocalization between viral pUL69 and cyclin T1 in four different human cell types. Cells were infected with HCMV strain AD169 or Merlin, fixed at late time points post-infection (HFF, 4 days; MRC-5 and ARPE-19, 6 days; TEV-1, 5 days) and analysed by indirect immunofluorescence and confocal microscopy (Feichtinger et al., 2011; Milbradt et al., 2010). Antibodies: polyclonal anti-cyclin T1 (sc-10750; Santa Cruz Biotechnology), monoclonal anti-UL69 (clone 69-66; Feichtinger et al., 2011).

(b) Coimmunoprecipitation (CoIP) analysis showing the interaction between pUL69 and cyclin T1 in HFFs infected with HCMV Merlin (m.o.i. 0.2, 6 days p.i.). CoIP was performed using antibodies against cyclin T1 and aldolase under the conditions described previously (Thomas et al., 2014; Webel et al., 2014; Graf et al., 2013). Quantitative values determined by densitometry (percentage CoIP cyclin T1) are given below the panels. Expression controls (Input) were taken prior to the addition of CoIP antibody and subjected to standard Western blot analysis; m, mock-infected.
pUL69 is not selectively directed to one single target residue, but appears to occur in a complex pattern, including potential sites scattered throughout the N-terminal portion of pUL69.

As a next step, the previously described phenomenon of intranuclear fine-speckled aggregation of pUL69 was analysed by the use of various kinase inhibitors. The initial report by Sanchez & Spector (2006) provided evidence that the CDK inhibitor roscovitine influences the intranuclear localization of pUL69 in HCMV-infected fibroblasts by changing pUL69 homogeneous nuclear distribution towards a speckled aggregation. In the present study, our data support and extend this notion, in that the formation of fine-speckled aggregates was not a strain-specific event, but rather a general property shared by different strains of HCMV, i.e. Merlin (Fig. 3a), AD169 (Fig. 3b) and TB40 (data not shown). Based on our reports that the speckled phenotype of pUL69 is induced by treatment with various kinase inhibitors (Rechter et al., 2009; Thomas et al., 2009; Feichtinger et al., 2011), we extend this feature to a recently identified selective inhibitor of CDK7, LDC4297 (Fig. 3b). LDC4297 is a highly potent compound that exerts broad antiviral activity at submicromolar to nanomolar concentrations [including against HCMV, EC50 (50% effective concentration) = 0.02 ± 0.00 μM; Hutterer et al., 2015]. As shown in Figs 3(b) and S2, LDC4297 induced the typical pattern of intranuclear fine-speckled accumulation of pUL69. The impact of CDK7 on pUL69 localization was further substantiated by a second CDK7-selective compound, LDC3140 (Kelso et al., 2014), inducing an identical pattern of speckled pUL69 aggregation (Fig. S2 panels q–t). Notably, the majority of pUL69 aggregates were found concentrated in...
Fig. 3. Formation of fine-speckled nuclear pUL69 aggregates. HFFs were used for infection with HCMV strains Merlin (a) or AD169 (b, c) at m.o.i. 0.5 and analysed as described in Fig. 1. (a, b) Induction of fine-speckled nuclear pUL69 aggregates (72 h p.i.) by three different CDK inhibitors (added at 24 h p.i.). (c) Quantitative evaluation of the phenotype of pUL69 localization in HCMV-infected cells. In each sample, at least triplicates of groups of 100 cells were counted under the microscope and data are given as mean±SD. Representative confocal images of spontaneous fine-speckles are presented for consecutive time points of infection (compare with Fig. S3A). Antibodies and compounds (see Fig. 1): monoclonal anti-MCP (Hamilton et al., 2014), monoclonal anti-pUL44 and polyclonal anti-UL97 (Webel et al., 2014), roscovitine (Rosco, 10 μM), R22 (10 μM; Feichtinger et al., 2011), LDC4297 (1 μM; Hutterer et al., 2015), GCV (20 μM).
viral replication centres (Fig. 3b and Fig. S2 for a costaining of pUL69 with the marker of replication centres pUL44, see Fig. S3B) and aggregates included a colocalization of viral kinase pUL97 (Fig. 3b, panels l–o). Interestingly, however, these aggregates did not contain cyclin H Fig. S2 panels x–y) and CDK7 only to some extent Fig. S2 panels v–y). This was in contrast to CDK9 and cyclin T1, which both perfectly colocalized with CDK inhibitor-induced pUL69 speckles. These data suggest that intranuclear pUL69 localization is regulated in a complex fashion and colocalization patterns vary for functionally different CDK–cyclin complexes.

As another regulatory aspect, to the best of our knowledge we provide the first evidence that fine-speckled nuclear pUL69 aggregation is a late-phase event of HCMV replication. For this purpose, R22 was used as an inducer of aggregation and subsequently ganciclovir (GCV) was used for cotreatment to restrict the number of HCMV-infected cells entering the late viral replicative phase. Late-phase replication was monitored by the expression of the true late major capsid protein (MCP). Notably, speckled pUL69 was detected at very low levels at early times [<0.1 %, 32 h post-infection (p.i.)], but steadily increased during later times of infection (between 60 and 90 % in individual experiments), and a drastic late-phase block of viral replication induced by increasing concentrations of GCV up to 20 μM substantially reduced the levels of speckled pUL69 (data not shown). When analysing the late phase of HCMV replication (72 h p.i.; Fig. 3c, lower panel), the levels of pUL69/MCP-positive cells were reduced to 9.7 ± 4.6 % under R22/GCV cotreatment, compared with cells infected in the absence of GCV (no inhibitor, 55.6 ± 24.3 %; R22, 26.9 ± 6.4 %). Importantly, amongst the group of pUL69/MCP-positive cells, the percentage of cells showing speckled pUL69 was similarly high under R22 treatment alone or R22/GCV cotreatment (90.8 ± 2.3 and 89.5 ± 3.8 %, respectively). This finding suggests that the formation of pUL69 speckled aggregates occurs during the late phase of HCMV replication.

Interestingly, our study also revealed a spontaneous formation of fine-speckled pUL69 aggregates in the absence of inhibitors in HCMV-infected primary fibroblasts. A quantitative evaluation of confocal images indicated that this spontaneous aggregation (seen at late time points of infection between 48 and 72 h) was a rare event occurring at a frequency of 0.44–1.00 % of cells. Spontaneous fine-speckling was qualitatively indistinguishable from the phenotype obtained by inhibitor treatment (Figs 3c, upper panel, and S3). So far, it remains speculative whether this pUL69 phenotype is important for viral replication and may be part of the phosphorylation-dependent regulation of pUL69 trafficking.

Combined, our data strongly underline that phosphorylation has a functional consequence on intranuclear localization and functionality of pUL69. It is important to stress that inhibition of CDK activity impairs the mRNA export activity of pUL69 as demonstrated previously (Rechter et al., 2009). We favour the idea that pUL69 phosphorylation is mediated through a cyclin-bridged association of viral and cellular kinases, eventually stimulating the activity of pUL69 as nucleo-cytoplasmic mRNA transport factor. Thus, the correct state of phosphorylation may determine both transport and activity of pUL69 so that a dysregulated pattern of phosphorylation (both hypo- or hyperphosphorylation) possibly results in an impairment of both properties. So far, however, the mechanism connecting pUL69 phosphorylation, localization and function-ality is not fully understood, so that details of this regulatory concept have to be determined by future studies.

Acknowledgements

The authors are grateful to all members of the laboratories of W. D. R., T. S. and M. M. for contributing to the ongoing cooperative studies. We greatly appreciate the supply of valuable reagents, i.e. Richard Stanton for the HCMV strain Merlin (Institute of Infection & Immunity, Cardiff University, Cardiff, UK), William Britt for monoclonal anti-Ul69 clone 69-66 (University of Birmingham, AL, USA) and Jan Eickhoff for CDK7 inhibitors (Lead Discovery Centre GmbH, Dortmund, Germany). This work was supported by Deutsche Forschungsgemeinschaft (SFB796 G3 to M. M., B3 to T. S., and DFG MA1289/6-1 and 8-1), Wilhelm Sander-Stiftung, Munich (2011.085.1-2 to M. M.), ELAN Fond University Erlangen-Nürnberg (J. M.-2012 and C. H.-2015), DAAD-Go8 (54390135 to M. M./W. D. R.) and IZKF University Erlangen-Nürnberg (J30 to M. T.).

References


