Cyclin-dependent kinases phosphorylate the cytomegalovirus RNA export protein pUL69 and modulate its nuclear localization and activity

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Replication of human cytomegalovirus (HCMV) is subject to regulation by cellular protein kinases. Recently, we and others reported that inhibition of cyclin-dependent protein kinases (CDKs) or the viral CDK ortholog pUL97 can induce intranuclear speckled aggregation of the viral mRNA export factor, pUL69. Here, we provide the first evidence for a direct regulatory role of CDKs on pUL69 functionality. Although replication of all HCMV strains were dependent on CDK activity, we found strain-specific differences in the amount of CDK inhibitor-induced pUL69 aggregate formation. In all cases analyzed, the inhibitor-induced pUL69 aggregates were clearly localized within viral replication centers, but not subnuclear splicing, pore complex or aggresome structures. The CDK9 and cyclin T1 proteins colocalized with these pUL69 aggregates, while other CDKs behaved differently. Phosphorylation analyses in vivo and in vitro demonstrated pUL69 was strongly phosphorylated in HCMV-infected fibroblasts and that CDKs represent a novel class of pUL69-phosphorylating kinases. Moreover, the analysis of CDK inhibitors in a pUL69-dependent nuclear mRNA export assay provided evidence for functional impairment of pUL69 under suppression of CDK activity. Thus, our data underline the crucial importance of CDKs for HCMV replication, and indicate a direct impact of CDK9/cyclin T1 on the nuclear localization and activity of the viral regulator pUL69.

Human cytomegalovirus (HCMV) is a member of the Herpesviridae family and a human pathogen with world-wide distribution. Primary HCMV infection of the immunocompetent host is usually asymptomatic, whereas severe disease can occur upon infection of the immunocompromised and immunonnaive. HCMV is a leading cause of complications in transplant recipients and AIDS patients, and congenital infection may result in mental impairment and hearing loss (1).

HCMV replication is differentially regulated in different host cell types, and viral replication is dependent on regulation of the cell cycle (2). HCMV infection induces cell cycle arrest; whilst simultaneously the virus sustains an active cellular metabolic state supporting productive infection (3). Infected cells arrest in a pseudo-G1 state with high levels of cyclin E and cyclin E-associated kinase activity (4-6). A number of additional alterations of cyclin-dependent protein kinase (CDK) activity have also been described, such as increased synthesis and reduced degradation of cyclin B1, as well as cytoplasmic translocation of CDK1 in HCMV-infected cells (7). The upregulation of CDK activity during HCMV replication implies that viral replication requires CDK activity to create an environment favourable for efficient viral transcription, genome replication and assembly of viral particles. Several regulatory steps in HCMV replication are dependent on CDK activity, particularly those involving CDK1, -2, -7 and -9 (8-12). Additionally, inhibition of CDK activity affects replication of HCMV and other herpesviruses (13). Roscovitine, a purine analog that preferentially inhibits CDK1, -2, -5, -7 and -9, has been shown to decrease viral DNA synthesis, production of late viral protein and infectious virus (8, 9, 12, 14). Roscovitine is therefore a useful tool to investigate the impact of CDK activity on viral replication, and understand interregulation between CDKs and viral proteins. Cross-talk between CDKs and other protein kinases during HCMV replication is one issue of current interest (15).

CDKs, particular serine/threonine kinases that become activated upon binding to cyclins, are involved in the regulation of multiple cellular processes. They can be subdivided into two major functional groups - cell cycle-associated CDKs and transcriptionally regulating CDKs. A prototype of the transcriptionally regulating CDKs is the positive transcription elongation factor b (P-TEFb), which is
composed of CDK9 and cyclin T1 (cycT1). This complex is an important regulator of transcription through phosphorylation of the C-terminal domain of the large subunit of RNA polymerase II (RNAII), thus allowing for transcription elongation (16). The expression of many genes is regulated at the level of transcription elongation, and the activity of the P-TEFb complex is tightly controlled. For example, the association of 7SK snRNA and HEXIM1 acts as an inhibitor to P-TEFb (17-20), whereas autophosphorylation of phospho-acceptor sites at the CDK9 C-terminus acts to stimulate and promote nuclear translocation of the P-TEFb complex (21).

Recently, it was demonstrated the HCMV-encoded protein kinase pUL97 has structural resemblance (22) and activities similar to CDKs, and thus represents a CDK ortholog (23-25). It was shown pUL97 phosphorylates and inactivates the retinoblastoma protein, stimulates cell cycle progression, and is insensitive to cellular CDK regulator proteins (23-25). It was shown pUL97 phosphorylates and inactivates the retinoblastoma protein, stimulates cell cycle progression, and is insensitive to cellular CDK regulator proteins that normally attenuate CDK activity (23). Overall, pUL97 is an important determinant of viral replication (25, 26). Previous studies reported that deletion of the UL97 region from the viral genome or pharmacological inhibition of pUL97 kinase activity drastically reduce viral replication (24, 27-33).

Amongst the viral proteins identified as substrates of pUL97, the pluripotent regulator pUL69, appears functionally relevant. pUL69 acts as a transcriptional activator (34, 35), a nuclear mRNA export factor (36) and a mediator of cell cycle arrest (37, 38). Recent studies show pUL69 binds RNA, has nucleocytoplasmic shuttling activity, and recruits the cellular mRNA export machinery via interaction with the cellular mRNA export factor UAP56/URH49. This latter activity promotes cytoplasmic accumulation of unspliced mRNA (36, 39, 40). pUL69 is a phosphoprotein subject to phosphorylation by the pUL97 viral kinase (41), although it is still unclear whether CDKs also play an important role in its phosphorylation. Against this background, it is significant that the CDK inhibitor roscovitine influences the intranuclear localization of pUL69 in HCMV-infected fibroblasts by changing pUL69 homogeneous nuclear distribution towards speckled aggregation (9).

In this report, we provide evidence for direct targeting of pUL69 by CDKs, which modulates pUL69 nuclear localization and activity. Findings in support of this concept are the speckled nuclear aggregation of pUL69 induced by CDK inhibitors, colocalization of CDKs and pUL69 in HCMV-infected cells and the direct in vitro phosphorylation of pUL69 by CDK/cyclin complexes.

**Experimental Procedures**

**Cyclin-dependent protein kinase (CDK) inhibitors and reference compounds**

Roscovitine was purchased from Calbiochem, Germany. CDK inhibitors A14, A43, A50, A79, A98, R25 and R58 (aminopyrimidines) were provided by GPC Biotech AG, Martinsried, Germany. The following reference compounds were also used: G06976 (inhibitor of pUL97 and protein kinase C), G67874 (inhibitor of protein kinase C) (28, 42), AG490 (tyrphostin) and ganciclovir (GCV). All compounds were prepared in DMSO, except for GCV in aqueous solution, and aliquots stored at -20 °C.

**Cell culture, HCMV infections and plasmid transfections**

HFFs (approximately 2.5x10^5 cells per well) were infected as described previously (46). Transfection of 293T cells was performed using polyethylenimine reagent (PEI; Sigma) as described previously (47).

**Indirect immunofluorescence analysis**

HFFs were grown on coverslips in 6-well plates (4x10^5 cells per well) and infected with HCMV laboratory strain AD169, or HCMV mutants GDGrXbaF4, GDGrP53 and 759rD100 at a multiplicity of infection (MOI) of 0.5 or 1.0. CDK inhibitors were added to the culture media either immediately after infection or 24 hours post infection (hpi). Culture media containing inhibitors were refreshed every 24 h. Cells were fixed with 4 % paraformaldehyde (10 min, room temperature) and permeabilized using PBS/0.2 % Triton-X-100 (20 min, 4 °C). Primary antibodies used were PAb-CDK1 (sc-954, Santa Cruz Biotechnology), PAb-CDK2 (sc-6248), PAb-CDK9 (sc-484), PAb-cycT1 (sc-10750), PAb-UL69 (34), PAb-UL54 (recovered from human antisera) and PAb-UL97 (kindly provided by D. Michels, Univ. Ulm, Germany); monoclonal antibodies used were MAb-CDK7
infected cells using the Wizard infection. Total DNA was extracted from virus-supernatant taken at various time points post genome equivalents in initial viral stocks and genome copies normalized against cellular plasmid pHM123 containing the IE1 cDNA (50).

DNA copies was prepared by serial dilutions of DNA standard for quantification of viral genome carboxytetramethylrhodamine quencher dye. The (5’-TCAGTGGAAGATGAAACATACGTTC-3’) together with a albumin gene-specific probe 3’ and 5’- GCATGGAAGGTGAATGTTTCAG-3’ gene locus (5’- GTGAACAGGCGACCATGCT-GAGCAGACTCTCAGAGGATCG-3’) and 5’ -AAGCGGCCTCTGATAACCAAG-3’ and 5’ -CATGCAGATCTCCTCAATGCGCGC-3’). To calculate the ratio of viral DNA per cellular region exon 4 (5’- CATGCAGATCTCCTCAATGCGCGC-3’). To calculate the ratio of viral DNA per cellular DNA equivalent, the cellular DNA was quantified in parallel using primers DNA equivalent, the cellular DNA was calculated the ratio of viral DNA per cellular

To quantify viral genome copies normalized against cellular genome equivalents in initial viral stocks and supernatant taken at various time points post infection. Total DNA was extracted from virus-infected cells using the Wizard® DNA Purification Kit (Promega, Mannheim, Germany). qPCR was performed in a 25 µl reaction mixture containing 5 µl of either the sample or the standard DNA solution. Additional components of the reaction mixture were 12.5 µl 2x TaqMan PCR Mastermix (Applied Biosystems), 7.5 pmol of each primer complementary to a region withing exon 4 of the IE1 gene locus (5’-AAGCGGCCTCTGATAACCAAG-3’ and 5’-GAGCAGACTCTCAGAGGATCG-3’) and 5 pmol of probe directed against the HCMV MIE gene locus (5’- GTGAACAGGCGACCATGCT-GAGCAGACTCTCAGAGGATCG-3’). Both probes used were labeled with 6-carboxyfluorescein reporter dye and 6-carboxypteremethylrhodamine quencher dye. The DNA standard for quantification of viral genome copies was prepared by serial dilutions of plasmid pHM123 containing the IE1 cDNA (50).

The cellular standard was composed of albumin PCR products obtained from lymphocyte cell extracts (51). The thermal cycling conditions consisted of two initial steps of 2 min at 50°C and 10 min at 95°C followed by 40 amplification cycles (15 see 95°C, 1 min 60°C). DNA extracts were analyzed in triplicate for each sample.

Yeast two-hybrid analysis- Protein interactions were analyzed using GAL4 fusion proteins (GAL4-BD, DNA binding domain; GAL4-AD, activation domain) in the yeast two-hybrid system as described previously (24). An expression plasmid for cycT1 was provided by L. Lania (52). Expression plasmids for CDK9 and cycT1::CDK9 were subcloned from pACTII-T1 and pRC/CMV-PITALRE-HA (52, 53). Saccharomyces cerevisiae strain Y153 was used for interactor analysis and the selection of clones achieved by cultivation on media restricting growth to combined tryptophan/leucine prototrophy. Selected colonies were analyzed for β-galactosidase activity by filter lift tests.

In vitro kinase assay (IVKA)-Recombinant CDK/cyclin complexes CDK1/cycB1, CDK2/cycE, CDK7/cycH/MAT1 and CDK9/cycT were purchased from ProQinase (Freiburg, Germany). As a putative substrate protein, pUL69 was expressed in 293T cells and cells were lysed in RIPA buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 1 % Na-desoxycholate, 0.1 % SDS, 0.5 % NP40, 10 mM NaF, 2 mM Na-orthovanadate, 1 mM PMSF, 10 µg/ml aprotinin) before pUL69 was immunoprecipitated using PAb- UL69 (34). After washing with HNTG buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 % glycerine and 0.1 % Triton X-100) and standard assay buffer (125 mM HEPES-NaOH pH 7.5, 7.5 mM MgCl2, 7.5 mM MnCl2, 7.5 µM Na-orthovanadate, 2.5 mM DTT) immunoprecipitates were incubated with 20 µl standard assay buffer, 1 µM ATP, 2.5 µCi [γ-33P]ATP and 10 µl recombinant CDK/cylin complex [50 ng of CDK2/cycE, 200 ng of CDK1/cycB1, 200 ng of CDK7/cycH/MAT1 or 200 ng of CDK9/cycT; each diluted in kinase-dilution-buffer (500 mM HEPES-NaOH pH 7.5, 2.5 mg/ml PEG2000, 10 mM DTT)] for 20 min at 30 °C. As a positive control for substrate phosphorylation, purified RB-CTF (C-terminal fragment of the human retinoblastoma protein) was added to reactions (1 µg). Reactions were stopped with SDS loading buffer and samples analyzed by SDS-PAGE and Western blot procedures. Radioactive signals were detected by

(5’-AAGCGGCCTCTGATAACCAAG-3’ and 5’-GAGCAGACTCTCAGAGGATCG-3’). Both probes used were labeled with 6-carboxyfluorescein reporter dye and 6-carboxypteremethylrhodamine quencher dye. The DNA standard for quantification of viral genome copies was prepared by serial dilutions of plasmid pHM123 containing the IE1 cDNA (50). The cellular standard was composed of albumin PCR products obtained from lymphocyte cell extracts (51). The thermal cycling conditions consisted of two initial steps of 2 min at 50°C and 10 min at 95°C followed by 40 amplification cycles (15 see 95°C, 1 min 60°C). DNA extracts were analyzed in triplicate for each sample.

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exposure to phosphoimager plates and quantified by AIDA software.

In vivo labeling assay- Proteins were labeled in vivo in HCMV-infected HFFs by incubation with $[^{32}P]$orthophosphate (150 µCi/4.5x10^5 cells) in the culture media. Cells were lyzed in RIPA buffer and supernatants subject to immunoprecipitation (PAb-UL69). Samples were analyzed by SDS-PAGE/Western blot as described for the in vitro kinase assay.

Nuclear mRNA export assay for pUL69- A nuclear mRNA export assay, based on the export activity of recombinantly expressed pUL69, was performed with lysates from transfected HeLa cells as described previously (39). CAT reporter assays were performed as described by Farjot et al. (54). The plasmid construct pDM128:CMV/RRE, encoding an intron-containing mRNA with the coding sequence of chloramphenicol acetyl transferase (CAT), was used as a reporter of nuclear export activity. CDK inhibitors were added 16 h post transfection. CAT protein expression was quantified following cell lysis at 48 h post transfection and analyzed in triplicate using a CAT enzyme-linked immunosorbent assay (Roche Molecular Biochemicals).

Results

HCMV replication is regulated by CDK activity: CDK inhibitors induce a pronounced aggregate formation of pUL69 that is quantitatively different for variants of HCMV. As described previously, the intranuclear localization of the viral regulator pUL69 is significantly altered in the presence of the CDK inhibitor roscovitine, with the formation of speckled nuclear aggregates induced in the late phase of replication (9). This phenomenon was detectable for several strains of HCMV such as Towne (9), AD169 (41; Fig. 1A) and clinical isolates (unpublished results, Rechter, S., and Marschall, M.). For strain AD169, roscovitine treatment produced pUL69 aggregation in 67.3 % of virus-positive cells (Fig. 1C). This phenotype varied in quantitative terms between AD169-derived virus variants GDGrXbaF4, GDGrP53 and 759rD100. As depicted in Fig. 1B, GDGrXbaF4 and 759rD100 contain a GCV resistance-conferring mutation in ORF UL97 (deletion 590-593) (44). GDGrP53 and 759rD100 contain a point mutation in ORF UL54 (A987G) resulting in cidofovir (CDV) resistance (43, 45) and high level GCV resistance (Fig. 1D, GCV). When analyzing the nuclear localization of pUL69, variants GDGrXbaF4 and GDGrP53 were similar to AD169 with pUL69 aggregate formation in the presence of roscovitine (Fig. 1A, a-d, f-i). However in quantitative terms, the pUL69 aggregation of GDGrP53 was significantly reduced (38 % of virus-positive cells, p<0.01; Fig. 1C) compared to the parental strain AD169. As shown for AD169 and the variants GDGrXbaF4 and GDGrP53, an additional CDK inhibitor, R58, also induced a pUL69 phenotype similar to that induced by roscovitine (Fig. 1A, k-n). R58 is a strong inhibitor of CDK2, CDK5 (IC50 <1 µM in vitro), and possibly other CDKs. The double-mutant 759rD100 behaved differently, showing very little speckled aggregation of pUL69 in infected cells treated with roscovitine or R58 (Fig. 1A, e, j, o). Quantitative immunofluorescence analysis demonstrated only 15% of 759rD100-infected fibroblasts showed pUL69 aggregates under roscovitine treatment, which represented a highly significant reduction (p<0.0001; Fig. 1C). Similar results were obtained with inhibitor R58. The UL97/UL54 mutant 759rD100 showed reduced replicative sensitivity towards roscovitine, demonstrated on plaque reduction assay (Fig. 1D, Rosco), but showed a strong sensitivity towards the second CDK inhibitor, R58. This indicated that R58, although inefficient in inducing pUL69 aggregates in 759rD100-infected cells, mediated an inhibitory effect on viral replication, albeit through a mode of action which seemed independent from pUL69.

The question whether observed differences between HCMV variants were due to major alterations in viral replication characteristics was addressed by quantitative real-time PCR (qPCR). HFFs were infected with DNA-normalized viral stocks (that is, viral DNA copy numbers per cell equivalents) and the kinetics of viral genomic DNA synthesis were determined as depicted in Fig. S1. The genomic replication curves of AD169 and GDGrXbaF4 were very similar, whereas 759rD100 and GDGrP53 showed a clear replication deficit throughout the period analyzed. This most likely can be attributed to the pUL54 DNA polymerase mutation of 759rD100 and GDGrP53. However, the qPCR data cannot fully explain the roscovitine-induced pUL69 aggregation phenotypes of the virus variants. While GDGrP53 showed the lowest replication efficiency, 759rD100, but not GDGrP53, showed a clear lack of pUL69 aggregate formation. Thus, the phenotype peculiarity of variant 759rD100, with its double mutation in pUL54 DNA polymerase and pUL97 protein kinase,
needs to be further analyzed on a mechanistic basis.

In this context, it was interesting to observe that a known pUL97 kinase inhibitor, Gö6976, but not an unrelated tyrosine kinase inhibitor, AG490, produced a pattern of speckled pUL69 aggregate formation very similar to roscovitine or R58. Parental AD169 and variants GDGrXbaF4 and GDGrP53 showed a pronounced Gö6976-induced pUL69 aggregation (Fig. S2, t, v, x), while very little aggregate formation was observed for 759rD100 in response to Gö6976 treatment (Fig. S2, z; statistically significant, p<0.01). Of note, pUL97 was never observed in colocalization with pUL69 aggregates but remained in a non-speckled, homogeneous nuclear distribution. Additionally, the presence of Gö6976 led to some exclusion of pUL97 from viral replication centers (Fig. S2, c, u), an effect that had been described before (55). Thus, inhibition of pUL97 as well as CDKs can induce the pUL69 aggregation phenotype in several variants of HCMV.

In order to narrow down the number of CDKs associated with the formation of pUL69 aggregates, a series of novel inhibitors with strong inhibitory potential against CDKs in vitro were utilized. These inhibitors fell into two groups with respect to their ability to produce pUL69 speckled aggregates: five compounds (A14, A43, A79, R25 and R58) induced strong effects comparable to roscovitine, whereas two other compounds (A50 and A98) failed to alter pUL69 distribution (Table S1). These CDK inhibitors all share a strong inhibitory potential against CDK1 and CKD2 in vitro (IC50 <1 µM) and possibly further inhibitory effects against other CDKs. Thus, the pattern of CDK inhibition required for pUL69 aggregation could not be deduced from this experiment. However, it is highly suggestive that CDK1/-2 inhibition is not sufficient to confer the phenotype of pUL69 aggregation and additional inhibitory activity is required.

**CDK inhibitor-induced intranuclear aggregates of pUL69 are localized within viral replication centers.** In order to characterize the speckled aggregation of pUL69 more closely, HCMV-infected fibroblasts were analyzed under roscovitine treatment by costaining of pUL69 with a selection of viral and cellular nuclear proteins (Fig. 2). Viral DNA polymerase pUL54 and its processivity factor pUL44 are prominent markers of viral replication centers and pUL69 is typically also detectable within these compartments. Following infection of HFFs with HCMV AD169 in the presence of roscovitine, pUL44 (Fig. 2, c and g) and pUL54 (data not shown) did not alter their localization but markedly, the speckles aggregates of pUL69 fully localized within the area of replication centers (Fig. 2, e-h). This suggests an accumulation of the replication center-associated fraction of pUL69 into subnuclear speckles under conditions of inhibited CDK activity. Additionally, further types of prominent intranuclear structures were examined, such as splicing compartments (marked by splicing factor SC-35), the nuclear pore complex (NPC, marked by NUP62/152/90) or aggresome structures (marked by heat shock cognate protein 70, HSC70 (S6)). As illustrated in Fig. 2, neither SC-35 (k and o), NUP62/152/90 (s and w) nor HSC70 (data not shown) displayed alterations in their localization or colocalized with pUL69.

**CDK inhibitor-induced aggregates of pUL69 colocalize with CDK9 and cyclin T1.** The investigation of CDK distribution patterns in HCMV-infected HFFs under inhibitor treatment showed a specific association of CDKs with pUL69 speckled aggregates (Fig. 3A and Fig. S3). CDK1, -2, -7 and -9 were analyzed under roscovitine, R58 or Gö6976 treatment. AG490 and Gö7874, which neither affect pUL97 nor CDK activity, served as specificity controls. Strikingly, CDK9 was found to undergo changes in localization similar to pUL69. CDK9 accumulated in replication centers of HCMV-infected cells as shown by colocalization with pUL69 in the absence of inhibitor (Fig. 3A, e-h). This colocalization was further developed in the presence of roscovitine or R58, that is, CDK9 also aggregated in the form of nuclear speckles (Fig. 3A, i-p). CDK9 aggregation was only marginally detectable in the presence of the pUL97 inhibitor Gö6976 (Fig. 3A, q-t). Both controls, Gö7874 (Fig. 3A, u-x) and AG490 (data not shown), had no impact on the localization of pUL69 and CDK9. In a next step, the regulatory subunit of CDK9, cyclin T1 (cycT1), was analyzed. As depicted in Fig. 3B, cycT1 was not only recruited to HCMV replication centers (e-h) but also formed speckled aggregates in colocalization with pUL69 under treatment with roscovitine (i-l) or R58, but not Gö6976 (data not shown). These findings strongly suggest that, although CDK inhibitors as well as pUL97 inhibitors induce a very similar speckled aggregation of pUL69, the composition of the structures and the underlying mechanisms are different. Thus, roscovitine-mediated aggregation is likely to be associated with CDK9/cycT1 activity, while Gö6976-
mediated aggregation appears to be CDK-independent.

Interestingly, closer investigation of protein-protein interactions by yeast two-hybrid analysis revealed direct interactions between pUL69 and cyclin T1 as well as a cyclin T1:CDK9 fusion construct, but not with CDK9 alone (Fig. 3C). Signal intensity of the positive scores of the filter lift staining remained at a moderate level compared to the positive control (CDK9 and cyclin T1) indicating a dynamic mode of low-affinity interaction between pUL69 and cyclin T1.

A putative colocalization between pUL69 and other CDKs was further analyzed. CDK7, functionally related to CDK9, accumulated in replication centers similar to CDK9 and cyclin T1 in HCMV-infected cells (Fig. S3, e-h). However, neither CDK nor pUL97 inhibitors induced speckled aggregation of CDK7 (Fig. S3, i-t). For CDK2, HCMV infection did not lead to an incorporation into viral replication centers or any other detectable changes in intranuclear localization. Also, the addition of inhibitors had no impact on the pattern of nuclear localization of CDK2 (data not shown). On the other hand, a previously described translocation of CDK1 from the nucleus to the cytoplasm was detected in HCMV-infected cells (7). Hence, colocalization between CDK1 and pUL69 was not detected in the presence or absence of inhibitors.

Phosphorylation of pUL69 in vitro and in vivo. The strong interregulation of CDKs with the nuclear localization of pUL69 initiated investigation of CDK-mediated phosphorylation of pUL69. To this end, in vitro kinase assays were performed to analyze the ability of recombinant CDK/cyc complexes (CDK1/cyclin B1, CDK2/cyclin E, CDK7/cyclin H/MAT1 and CDK9/cyclin T) to phosphorylate pUL69 immunoprecipitated from transfected 293T cells. The activity of the CDK/cyc complexes were confirmed via phosphorylation of a reference substrate, RB-CTF (data not shown). Importantly, a clear signal for direct phosphorylation of pUL69 by CDK1/cyclin B1, CDK7/cyclin H/MAT1 and CDK9/cyclin T was detected (Fig. 4A, upper panel, lanes 1, 3 and 4). Nonspecific phosphorylation activity was excluded by the lack of measurable phosphorylation of HCMV pUL26, used as a specificity control (Fig. 4A, lower panel). Low-level baseline phosphorylation of pUL69 could be detected without addition of CDK/cyc, which probably indicates traces of pUL69-phosphorylating kinase activity in the immunoprecipitates (Fig. 4A, upper panel, lane 5). The fold increase in phosphorylation of pUL69 by CDK/cyc complexes was then determined via densitometry. As shown in Fig. 4B, the strongest phosphorylation was mediated by CDK1/cyclin B1 (12.4-fold increase). Pronounced levels of phosphorylation were also measured for CDK9/cyclin T (7.2-fold) and CDK7/cyclin H/MAT1 (6.4-fold), while CDK2/cyclin E-mediated phosphorylation was lower (4.1-fold). Thus, these in vitro data indicate that direct phosphorylation of pUL69 can be mediated by several CDK/cyc complexes.

To assess phosphorylation of pUL69 in vivo, we infected HFFs with HCMV AD169 for 2 days and incubated cells with [γ-32P]orthophosphate to allow for in vivo labeling of proteins. An evaluation of pUL69 immunoprecipitated from these cells revealed a strong signal of phosphorylation (Fig. 4C). Phosphorylation could be partly inhibited by the treatment of infected cells with 15 µM of roscovitine (signal reduction of approximately 66±13%, data not shown). However, this inhibition of phosphorylation did not occur in a CDK-specific manner and was also observed for other protein kinase inhibitors. This points to a complex regulation of the phosphorylation of pUL69. Thus, the in vivo phosphorylation of pUL69 appears to be dependent on CDK and other protein kinase activities.

The particularly strong CDK1/cyclin B1-mediated phosphorylation of pUL69, as demonstrated by in vitro data (Fig. 4A-B), raised questions about the nucleo-cytoplasmic translocation of CDK1. We determined whether CDK1 was detectable in pUL69-positive nuclei of HCMV-infected fibroblasts by performing immunofluorescence analysis including confocal laser-scanning microscopy. For this purpose, a kinetic study was performed to investigate localization patterns during the immediate early and early phases of HCMV replication (Fig. S4). Nuclear pUL69 was observed from 4 hpi and the percentage of pUL69-positive cells increased continuously over time. Under roscovitine treatment, a transient delay of pUL69 expression was detected (Fig. S4A), with reduced immunofluorescence signal intensities confirming a slightly lower level of pUL69 production. Interestingly, the beginning of nucleo-cytoplasmic translocation of CDK1 was observed at 8 hpi in both roscovitine-treated and untreated cells (Fig. S4B). Over the period analyzed, the fraction of pUL69-positive cells showing CDK1 in a nucleo-cytoplasmic or cytoplasmic localization steadily increased, with
a completion of the translocation at about 24 hpi. Of note, this translocation was slightly retarded through the inhibition of CDK activity by roscovitine. Thus, although a direct colocalization between CDK1 and pUL69 was not detectable, the presence of both CDK1 and pUL69 in the nuclei of HCMV-infected fibroblasts may allow an interregulation of the two proteins at early time points of infection.

Inhibition of CDK activity reduces mRNA export activity of pUL69. A nuclear mRNA export assay was performed to investigate functional aspects of pUL69. This assay determined the ability of pUL69 to export intron-containing CAT (chloramphenicol acetyl transferase) mRNA. As shown in Fig. 5, a decline in nuclear export activity was observed when CDKs were inhibited by either roscovitine or R58. The pUL69-mediated export signal was reduced to 43 % under roscovitine treatment (statistically significant, p<0.01) and to 63 % under R58 treatment. An inhibitory effect on the pUL69 nuclear export function was also detected for the pUL97-directed inhibitor, Gö6976 (41). Thus, CDK as well as pUL97 activity is required for the full functionality of pUL69 with regards to mRNA export.

Discussion

The HCMV replication strategy has evolved to an elaborate interregulation with factors controlling the cell cycle. On the one hand, HCMV ensures that the regulatory state of the cellular environment efficiently supports viral reproduction and, on the other hand, HCMV reprograms the cellular factors such as regulatory protein kinases from their original function towards virus-specific regulatory pathways (2, 3). A number of studies have shown that HCMV replication is functionally linked with CDK activity at various regulatory junctures. In this report, we provide novel insights into the link between cellular CDK activity and the intranuclear localization and functionality of the viral mRNA export factor pUL69. Our findings indicate: (i) HCMV-infected fibroblasts treated with CDK inhibitors show an intranuclear speckled aggregation of pUL69; (ii) variants of HCMV are differentially sensitive to inhibitors inducing pUL69 aggregation; (iii) speckled pUL69 aggregates are mainly localized within viral replication centers; (iv) CDK9 and cyclin T1 strictly colocalize with the inhibitor-induced speckled aggregates while other CDKs behave differently; (v) the HCMV-triggered nucleo-cytoplasmic translocation of CDK1 does not exclude a putative early nuclear interaction with pUL69; (vi) pUL69 is phosphorylated in vivo and in vitro, identifying CDKs (mainly CDK1 and -9) as novel pUL69-phosphorylating kinases; and (vii) CDK activity is required to stimulate a high level of nuclear mRNA export activity of pUL69 in a reporter assay.

The importance of CDK activity in the replication cycle of HCMV has been well documented (3, 4, 6-9, 11, 12). However, for most of these investigations, the description of molecular mechanisms linking CDK activity with viral regulation of replication were still unresolved. We have identified the viral regulatory protein pUL69 as one target of CDK-mediated regulation. This protein contributes to an HCMV-induced cell cycle arrest which may result from interaction with CDKs and/or cyclins but is poorly understood so far (37, 38). Additionally, pUL69 acts as a transcriptional transactivator via interaction with the cellular transcription elongation factor hSPT6 (57, 58) and as a nuclear RNA export factor via interaction with UAP56, a component of the cellular mRNA export machinery (36, 39, 40). As pUL69 is a phosphoprotein (35, 57) it has been speculated that its activity might be partly regulated through phosphorylation. In this report, we provide evidence for the phosphorylation of pUL69 by CDKs. pUL69 acted as a specific substrate in in vitro kinase reactions with all four analyzed CDKs (CDK1, -2, -7 and -9), whereby CDKs 1 and 9 exerted the highest pUL69-phosphorylating activity. Our data point to a combined impact of more than one CDK on the phosphorylation and activity of pUL69, based on findings that CDK1/cycB1 and CDK9/cycT exerted main activities in a pUL69-specific in vitro kinase assay while CDK9/cycT exclusively showed colocalization with pUL69 during late phase of infection. In addition, both CDK inhibitors roscovitine and R58 induced speckled aggregates of pUL69, although they possess partly different inhibitory profiles towards individual CDKs. Thus, it remains speculative which of the analyzed CDKs are the key determinants for pUL69 regulation. The impact of CDKs on HCMV replication may be an ordered sequence of events with pUL69-directed activity of CDK1 an early event during viral replication. Consistent with this, both pUL69 and CDK1 were localized in the nucleus prior to CDK1 nucleo-cytoplasmic translocation. This illustrates that CDK1, although not colocalizing with pUL69, may contribute to the regulatory phosphorylation of pUL69 at early time points of
infection. In contrast, CDK9 might be required at later time points for regulation of pUL69 activity, as demonstrated by direct colocalization of CDK9/cycT and pUL69 in late-phase replication centers. Integrating these findings, this indicates a regulatory impact of CDK9, and possibly further CDKs on the functionality of pUL69.

Interestingly, the pUL97 viral protein kinase was characterized as a CDK-related kinase possessing similar functional properties (23). Our studies of other protein kinases involved in the phosphorylation of pUL69 was compatible with these findings. Recently, we provided evidence the CDK ortholog pUL97 phosphorylates pUL69 (41). Combined with the data of the present study, we hypothesize that cellular CDK and viral pUL97 activity is required to modulate the nuclear localization and function of pUL69 during cytomegalovirus replication.

There is only a limited number of examples describing functional cross-talk between CDKs and herpesviral protein kinases. One prominent example is the sequentially ordered interregulation between CDK1/cdc25C and herpes simplex type 1 (HSV-1)-encoded protein kinases UL13 and US3 (59, 60). In this case, the two HSV kinases, UL13 and US3, are capable of phosphorylating, and thereby activating the regulatory cellular phosphatase cdc25C, which normally activates CDK1 (cdcc2) by removing two inhibitory phosphates. However, in HSV-1-infected cells the function of cdc25C is reduced to modulating CDK1 function. In infected cells, CDK1 acquires a new binding partner, the HSV DNA polymerase processivity factor UL42. The CDK1-UL42 complex then functions as an activator of late viral gene expression. In addition, CDK1 is able to phosphorylate UL42 and therefore possibly stimulate its activity in the UL42-DNA polymerase complex (61).

In summary, our data imply that CDK-specific regulatory pathways modulate the multiple functions of pUL69 in HCMV-infected fibroblasts. Further studies will be required to gain a deeper insight into the respective molecular mechanisms and to learn more about contact points of CDK-HCMV interaction.

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Footnotes

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FIGURE 4: Direct phosphorylation of pUL69 by CDK/cyc complexes in an *in vitro* kinase assay (A, B) and detection of phosphorylated pUL69 in HCMV-infected fibroblasts (C). (A) Recombinant pUL69 and pUL26 (FLAG-tagged) were expressed in 293T cells and immunoprecipitated with indicated antibodies following lysis at 72 h post transfection. Phosphorylation reaction was started by adding recombinant CDK/cyc complexes in the presence of radiolabelled \( [\gamma^{32}P]ATP \) to the precipitated proteins. (B) The increase in CDK/cyc-mediated phosphorylation of pUL69 in relation to basal levels was determined by means of densitometry. —, control w/o kinase. (C) HFF were infected with HCMV AD169 (MOI 0.5) for 2 days followed by an overnight *in vivo* labeling of proteins. Lysates were used for immunoprecipitation of pUL69 (PAb-UL69). Immunoprecipitates were subjected to SDS-PAGE/Western blotting to detect phosphorylated pUL69 (upper panel) and total pUL69 (lower panel).

FIGURE 5: Inhibition of CDK activity impairs the mRNA export activity of pUL69. HeLa cells were transfected with a pUL69-expressing plasmid in addition to the reporter plasmid (unspliced mRNA). The effect of the CDK inhibitors (added 16 h post transfection) on the activity of pUL69 to export CAT mRNA was determined after lysis of the cells 48 h post transfection by the CAT enzyme-linked immunosorbent assay. Statistical significance was calculated by student’s *t*-test.

SUPPLEMENTAL FIGURE S1. Growth analysis of GCV-resistant mutants compared to AD169. HFFs were infected with viral inoculi that were normalized to an equal uptake of viral DNA and cells were harvested at different time points as indicated. HCMV-specific qPCR was carried out to quantify viral genomes during the replicative cycle of GDGrXbaF4, GDGrP53 and 759rD100 in comparison to AD169.

SUPPLEMENTAL FIGURE S2: Analysis of speckled aggregates of pUL69 in the presence of pUL97 inhibitor Gö6976. HFFs were infected with HCMV AD169, GDGrXbaF4, GDGrP53 or 759rD100 (MOI 0.5) and 24 hpi medium supplemented with protein kinase inhibitors AG490 or Gö6976 was refreshed. Indirect immunofluorescence analysis was performed 72 hpi via detection of pUL69 and pUL97 with MAb 69-66 antibody and PAb-UL97 antibody.

SUPPLEMENTAL FIGURE S3: CDK7 colocalizes with pUL69 in viral replication centers but not in inhibitor-induced speckled aggregates. HFFs were infected with AD169 and treated with inhibitors as described for Fig. 5. Immunofluorescence staining of pUL69 (PAb-UL69) and CDK7 (MAb-CDK7) was performed as indicated at 72 hpi.

SUPPLEMENTAL FIGURE S4: Early kinetics of pUL69 production in HCMV-infected cells. (A) HFFs were infected with HCMV AD169 (MOI 1) and cultivated in the presence or absence of roscovitine and harvested at the time points indicated. Immunofluorescence staining of pUL69 was performed by means of MAb-UL69. (B) The nucleo-cytoplasmic translocation of CDK1 in HCMV-infected, pUL69-positive cells was analyzed by quantitative immunofluorescence analysis at early time points of HCMV replication. A mean ± SD of determination in quadruplicate is given (HCMV infection in duplicate, microscopic counting in duplicate).
Cyclin-dependent kinases phosphorylate the cytomegalovirus RNA export protein pUL69 and modulate its nuclear localization and activity
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