Recruitment of cyclin-dependent kinase 9 to nuclear compartments during cytomegalovirus late replication: importance of an interaction between viral pUL69 and cyclin T1

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Cyclin-dependent protein kinases (CDKs) are important regulators of cellular processes and are functionally integrated into the replication of human cytomegalovirus (HCMV). Recently, a regulatory impact of CDK activity on the viral mRNA export factor pUL69 was shown. Here, specific aspects of the mode of interaction between CDK9/cyclin T1 and pUL69 are described. Intracellular localization was studied in the presence of a novel selective CDK9 inhibitor, R22, which exerts anti-cytomegaloviral activity in vitro. A pronounced R22-induced formation of nuclear speckled aggregation of pUL69 was demonstrated. Multi-labelling confocal laser-scanning microscopy revealed that CDK9 and cyclin T1 co-localized perfectly with pUL69 in individual speckles. The effects were similar to those described recently for the broad CDK inhibitor roscovitine. Co-immunoprecipitation and yeast two-hybrid analyses showed that cyclin T1 interacted with both CDK9 and pUL69. The interaction region of pUL69 for cyclin T1 could be attributed to aa 269–487. Moreover, another component of CDK inhibitor-induced speckled aggregates was identified with RNA polymerase II, supporting earlier reports that strongly suggested an association of pUL69 with transcription complexes. Interestingly, when using a UL69-deleted recombinant HCMV, no speckled aggregates were formed by CDK inhibitor treatment. This indicated that pUL69 is the defining component of aggregates and generally may represent a crucial viral interactor of cyclin T1. In conclusion, these data emphasize that HCMV inter-regulation with CDK9/cyclin T1 is at least partly based on a pUL69–cyclin T1 interaction, thus contributing to the importance of CDK9 for HCMV replication.

INTRODUCTION

Human cytomegalovirus (HCMV) is a worldwide human pathogen belonging to the beta subgroup of the family Herpesviridae. Although primary HCMV infection of the immunocompetent host typically remains asymptomatic, severe disease can occur upon infection of immunonaïve and immunocompromised individuals such as neonates, transplant recipients and cancer or AIDS patients (Mocarski et al., 2007). The efficiency of HCMV replication is dependent on the interplay between viral and host-cell functions (Marschall et al., 2011). In particular, several regulatory steps in the course of HCMV infection depend on the activity of cellular cyclin-dependent protein kinases (CDKs), mainly CDK1, -2, -7 and -9 (Bain & Sinclair, 2007; Fortunato et al., 2000; Jault et al., 1995; Kapasi & Spector, 2008; Kapasi et al., 2009; Rechter et al., 2009; Sanchez & Spector, 2006; Sanchez et al., 2004; Tamrakar et al., 2005). HCMV-induced upregulation of CDK1, -2, -7 and -9 along with the corresponding cyclin subunits is explained by the fact that CDK activity is required at various stages of virus replication, such as the regulation of gene expression and the modification as well as localization of viral proteins (Bain & Sinclair, 2007; Kapasi et al., 2009; Rechter et al., 2009). CDKs are heterodimeric serine/threonine kinases that become activated upon binding to their regulatory cyclin subunits. CDKs are involved in the regulation of multiple cellular processes and can be subdivided into two major functional groups: cell cycle-associated and transcription-regulating CDKs. Whilst CDK1 and -2 mostly participate in cell-cycle regulation, CDK7 and -9 represent prototypes of transcription-regulating CDKs, which act by phosphorylating the C-terminal domain (CTD) of the large subunit of RNA.
polymerase II (RNAPII; Majello & Napolitano, 2001; Reinberg et al., 1998). In humans, the CTD consists of 52 repeats of the consensus heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser and is susceptible to differential phosphorylation levels in the event of transcription (Meinhart et al., 2005). Transcriptional activity is highly dependent on the phosphorylation pattern of the serines at positions 2 and 5. Once the hypophosphorylated form of RNAPII has been recruited to the pre-initiation complex, phosphorylation of the CTD at the serine residue at position 5 (mediated by CDK7/cycH/MAT-1, which is part of the transcription factor II H transcription complex) promotes the initiation of transcription. However, productive transcription requires further phosphorylation of CTD at serine residue 2 (mediated by CDK9/cyclin T1, also known as positive transcription elongation factor b).

Virus-specific regulation and reprogramming of CDK activity has been demonstrated by numerous studies on HCMV and other herpesviruses (Biglione et al., 2007; Dai-Ju et al., 2006; Davido et al., 2003; Diwan et al., 2004; Jaut et al., 1995; Kapasi et al., 2009; Kapasi & Spector, 2008; Kudoh et al., 2003, 2004; Pumfery et al., 2006; Rechter et al., 2009; Salvant et al., 1998; Sanchez & Spector, 2006; Sanchez et al., 2003, 2004; Schang, 2002, 2005a, b; Tamrakar et al., 2005; Wang et al., 2001). These findings have intensified the research on pharmacological CDK inhibitors (PCIs) and their putative use in antiviral therapy (Bresnahan et al., 1997; Herget & Marschall, 2006; Marschall & Stamminger, 2009; Schang et al., 2006). Classical PCIs are low-molecular-mass compounds (<500 Da) that consist of flat hydrophobic heterocyclic compounds. They typically bind by hydrophobic interactions and hydrogen bonds to the target kinases (Hardcastle et al., 2002), resulting mostly in binding of the ATP-binding pocket of their target CDKs, thus inhibiting kinase activity by competing with the ATP co-substrate. In many HCMV-based studies, the CDK inhibitor roscovitine has been proven as a useful tool to investigate virus–CDK interactions in detail. Roscovitine is a purine analogue classified as a PCI preferentially inhibiting CDK1, -2, -5, -7 and -9 (de la Fuente et al., 2003; Fischer & Gianella-Borradori, 2003; Schang, 2002, 2005b) that, interestingly, binds with highest affinity to the active form of target CDKs (De Azevedo et al., 1997; Knockaert et al., 2002).

For HCMV, studies using roscovitine have clearly demonstrated inhibition of virus replication at several stages. As the time point of drug addition is relevant, it is possible to distinguish between effects due to the presence of roscovitine immediately after infection and due to addition of the drug at 6–8 h post-infection (p.i.) (Kapasi & Spector, 2008; Kapasi et al., 2009; Sanchez & Spector, 2006; Sanchez et al., 2004; Tamrakar et al., 2005). Instant inhibition of CDK activity strongly impairs the entire immediate-early (IE) gene expression (mRNA and protein levels) with subsequent consequences on the expression of early and late genes. When the addition of roscovitine is delayed until 6–24 h p.i., other effects are observed, including a specific decrease in IE2 protein (IE2p86) and the major tegument protein, pp150. In addition, pUL69, a viral mRNA export factor and multifunctional protein, accumulates in a hyperphosphorylated form, in which its localization changes from a homogeneous distribution within viral nuclear replication centres towards a speckled aggregation throughout the nucleus (Rechter et al., 2009; Sanchez & Spector, 2006; Tamrakar et al., 2005). pUL69 has been characterized as a nuclear mRNA export factor (Lischka et al., 2001, 2006; Toth et al., 2006), a transcriptional activator (Winkler & Stamminger, 1996; Winkler et al., 1994) and an inducer of cell-cycle arrest (Hayashi et al., 2000; Lu & Shenk, 1999). Previously, we demonstrated that pUL69 is phosphorylated by CDKs (Rechter et al., 2009) and by viral protein kinase pUL97 (Thomas et al., 2009). Phosphorylation is a determinant of the mRNA export activity of pUL69, as concluded from the finding that both CDK and pUL97 inhibitors can suppress this activity in a reporter assay (Rechter et al., 2009; Thomas et al., 2009). In the present study, we characterized the interaction between CDK9/cyclin T1 and pUL69 and analysed the significance of this regulatory association. A scenario addressing the molecular mechanism and functional consequences of this mutual virus–cell inter-regulation is discussed.

RESULTS AND DISCUSSION

HCMV replication is positively regulated by CDK9 activity

The influence of CDK9 activity on HCMV replication was analysed by the use of a novel, selective CDK9 inhibitor termed R22. This compound, belonging to the chemical class of aminopyrimidines, showed a marked selectivity towards CDK9/cycT1 activity in vitro (IC50 0.04 ± 0.01 μM). Other CDK/cyclin complexes showed little sensitivity to R22 (Fig. 1a). A selectivity panel with a series of non-CDK human protein kinases illustrated the selective inhibitory potency of R22, with the exceptions of GSK-3β and EGFR, which indicated some additional modest level of inhibition (Fig. 1b). When assayed for a putative anti-cytomegaloviral activity, R22 exerted a concentration-dependent inhibition of HCMV replication in primary human foreskin fibroblasts (HFFs; Fig. 1c), as expressed by an IC50 value of 6.07 ± 0.38 μM. It should be noted that the IC50 of 0.04 μM for CDK9 in vitro was measured in a biochemical assay at the Km [ATP] of ~30 μM. Under intracellular conditions, R22 has competed with much higher concentrations of ATP in a range between 1 and 5 mM, meaning that the intracellular CDK9 inhibition with a measured IC50 of 6.07 μM is in the expected range (according to the Cheng–Prusoff equation, predicting a micromolar efficacy range in vivo; Cheng & Prusoff, 1973). This finding suggests a strong impact of CDK9 on the replication efficiency of HCMV. As far as the controversially discussed role of EGFR in HCMV entry and replication is concerned (Chan et al., 2009; Isaacson et al., 2007; Marschall & Stamminger, 2009), we cannot fully
Fig. 1. Anti-HCMV effect of CDK9 inhibitor R22 and inhibitor-induced nuclear relocalization of pUL69 and CDK9. The CDK9 inhibitor R22 was analysed for selectivity among CDK/cyclin complexes (a) or other human protein kinases (b). Data from the in vitro kinase assays are presented as IC$_{50}$ values or the percentage of inhibition at 10 μM (% no inhibitor). Assays were performed at least in triplicate or reproduced by independent confirmation settings. Grey-shaded fields, inhibition $\geq 50\%$ (cut-off); yellow-shaded fields, inhibition $\geq 90\%$. CDK/cyc, cyclin-dependent protein kinase/cyclin complex; Pim-1, proviral insertion site serine/threonine kinase 1; PLK1, polo-like kinase 1; v-Abl Gag, tyrosine kinase Abl fused to Mo-MuLV Gag; p70S6K, 70 kDa ribosomal serine/threonine protein S6 kinase; c-Met, receptor tyrosine kinase Met; c-Src, Src-family tyrosine kinase; c-Raf, Raf-1; SRPK1, serine/arginine-specific protein kinase; RSK1, ribosomal S6 kinase 1; ROCK2, Rho serine/threonine kinase 2; PDGFRβ, platelet-derived growth factor receptor β; ERK1, extracellular signal-regulated kinase 1; Kit, tyrosine kinase c-kit; Jnk1α1, c-Jun N-terminal kinase 1α1; InsR, insulin receptor; IkKβ, IκB kinase β; GSK-3β, glycogen synthase kinase 3β; EGFR, epidermal growth factor receptor; CK1α, casein kinase 1α; Akt/PKBα, serine/threonine kinase, also known as protein kinase Bα. (c) HFFs were infected with HCMV AD169 (m.o.i. of 0.25) and treated immediately with R22 at different concentrations as indicated. Cells were lysed at 7 days p.i. and subjected to GFP fluorometry. Determinations were performed in triplicate. Ganciclovir (GCV) served as an antiviral reference drug. (d) HFFs were infected with HCMV AD169 (m.o.i. of 1) and treated with roscovitine (Rosco; 10 μM) or R22 (10 μM), both added at 24 h p.i. Intracellular localization of pUL69 and CDK9 was determined by indirect immunofluorescence analysis at 72 h p.i. Nuclei were stained with DAPI.
exclude an additional EGFR-based anti-cytomegaloviral effect of R22. However, our previous studies with established EGFR inhibitors (e.g. AG1478) indicated that a pronounced anti-cytomegaloviral effect of these inhibitors can only be measured after pre-incubation of the cells prior to virus addition (Herget et al., 2004). In the experiment described in Fig. 1(c), R22 was not pre-incubated but was added immediately after virus adsorption, therefore arguing against an EGFR-based mode of inhibition. In addition, according to the Cheng–Prusoff equation, all other kinases analysed are predicted to possess a low affinity to R22, which renders it very unlikely that other kinases are responsible for the antiviral effect caused by R22. This issue was addressed further by supplementary experiments investigating the CDK9 selectivity of R22 (e.g. analyses on putative R22 effects towards the CDK1-mediated phosphorylation of E53 Thr-297 as well as CDK2- and CDK4/6-mediated substrate phosphorylations), not providing any indication for off-target effects of R22 (J. Eickhoff, unpublished data). Thus, inhibition of CDK9 activity was considered to be responsible for the R22-mediated block of HCMV replication.

### Nuclear speckled aggregates are formed in the presence of CDK9 inhibitor R22

The intracellular localization of CDK9 in HCMV-infected fibroblasts was analysed by indirect immunofluorescence using a confocal laser-scanning microscope. In particular, we wanted to clarify whether the inhibitor R22 was able to induce speckled aggregates containing viral pUL69, CDK9 and other proteins, as typically obtained by treatment with roscovitine (Rechter et al., 2009; Sanchez & Spector, 2006). As an important finding, R22 was as efficient as roscovitine in inducing speckled aggregates (Fig. 1d). HCMV-infected cells treated with R22 or roscovitine showed the typical speckled co-localization between pUL69 and CDK9 (Fig. 1d, panels i–p). In the absence of inhibitor, CDK9 was detected in a homogeneous localization over the entire nucleus in mock-infected cells (Fig. 1d, panels a–d) or was recruited into virus replication centres during late times of HCMV replication (Fig. 1d, panels e–h), as described previously (Rechter et al., 2009). In control settings, inhibitors of other protein kinases (including EGFR inhibitor AG1478) did not induce alterations of the nuclear localization of pUL69 or CDK9 (Rechter et al., 2009; Thomas et al., 2009; S. Feichtinger & M. Marschall, unpublished data). Thus, inhibition of CDK9 disturbed the normal nuclear localization of viral and cellular proteins such as pUL69 and CDK9 and thus may interfere with an important step of virus–cell inter-regulation.

### Direct interaction between pUL69 and cyclin T1

The strict co-localization patterns observed suggested an interaction between pUL69 and the CDK9/cyclin T1 complex. This idea was supported by previously published data obtained with a yeast two-hybrid system indicating a pUL69–cyclin T1 interaction (Rechter et al., 2009). To validate these findings, co-immunoprecipitation analyses were performed with proteins from HCMV-infected primary fibroblasts and plasmid-transfected 293T cells (Fig. 3). In the transient transfection system, all three proteins could be overexpressed to amounts suitable for this analysis; however, some co-expressions clearly influenced each other, leading to variations in expression levels (Fig. 3b). Importantly, the amounts of effectively immunoprecipitated proteins were nevertheless very similar among samples, as shown for the example of pFLAG-UL69 (Fig. 3a, lanes 2 and 3 and 7–9). Detection of the proteins was achieved by the use of tag-specific antibodies or antibodies recognizing both the recombinant and the endogenous version of the protein (pAb-cyc T1). As a striking result, cyclin T1, either in the absence or presence of co-expressed CDK9–haemagglutinin (HA), could be co-immunoprecipitated with an antibody recognizing pFLAG-UL69 (Fig. 3a and b, lanes 8 and 9). CDK9–HA alone was not co-immunoprecipitated together with pFLAG-UL69 (Fig. 3a, lane 7). All controls (Fig. 3a, lanes 1–6 and 10–13) demonstrated the reliability and specificity of the assay. In the reciprocal setting, transiently expressed pFLAG-UL69 could also be co-immunoprecipitated when using a precipitation antibody recognizing endogenous cyclin T1 (pAb-cyc T1; see Supplementary Fig. S1, available in JGV Online). Thus, pUL69 specifically binds to cyclin T1 in transiently transfected cells.

This result was confirmed with proteins co-immunoprecipitated from lysates of HCMV-infected cells. HFFs were
infected with HCMV AD169 (m.o.i. of 1 and 2) and subsequently treated with roscovitine or left untreated (Fig. 3c, d). In both cases, pUL69 could be co-immunoprecipitated with cyclin T1. Roscovitine treatment augmented the amount of co-immunoprecipitated pUL69 to some extent (Fig. 3c, upper panel, lanes 4 and 6). Cyclin T1 and CDK9 were found to be upregulated in HCMV-infected cells. Interestingly, CDK9 was to some extent also detectable in the cyclin T1–pUL69 co-immunoprecipitates (Fig. 3c, second panel, lanes 3–6). Due to the upregulation of CDK9, however, the experiment did not allow us to conclude whether this interaction of the three proteins was intensified in a non-competitive way in HCMV-infected compared with mock-infected cells. Nevertheless, the data suggest the formation of a functional three-component complex on the basis of a direct pUL69–cyclin T1 interaction.

The domain of pUL69 responsible for interaction with cyclin T1 was narrowed down by experiments using the yeast two-hybrid system (Fig. 3e). The pUL69–cyclin T1 interaction was detectable in independent experiments, but the signal intensity in filter lift assays was low so that most of the positive colonies remained close to the detection limit (Fig. 3e, panels 4, 7 and 11). Interestingly, when using N-terminally truncated versions of pUL69, the interaction was positive for aa 269–744 (Fig. 3e, panels 4, 7 and 11). However, for aa 380–744 (Fig. 3e, panels 9 and 10). In addition, a C-terminally truncated construct (aa 1–487; Fig. 3e, panels 11 and 12) also showed cyclin T1 interaction activity. Further C-terminally truncated versions of pUL69 were negative (data not shown). This result indicated the importance of pUL69 region aa 269–487 for cyclin T1 interaction.

**RNAPII is associated with virus replication centres and CDK inhibitor-induced nuclear aggregates**

The association of pUL69–cyclin T1–CDK9 aggregates with further cellular proteins was investigated. As the CDK9–cyclin T1 complex is a regulatory component of transcription complexes, the question of an association with RNAPII was addressed. For this purpose, RNAPII-
Fig. 3. Co-immunoprecipitation (CoIP) and yeast two-hybrid analyses demonstrating the interaction between CDK9, cyclin T1 and pUL69. (a, b) CoIP with proteins from transiently transfected cells: 293T cells were co-transfected with CDK9-HA, cyclin T1 (cycT1) and pFLAG-UL69 in various combinations as indicated and lysed at 48 h post-transfection. (a) pFLAG-UL69 was immunoprecipitated from cell lysates followed by detection of possible co-precipitated interactors by SDS-PAGE and Western blot (WB) analysis using tag- or protein-specific antibodies. (b) Expression of individual proteins was controlled. RFP, Red fluorescent protein. (c, d) CoIP with proteins from HCMV-infected cells: interaction between cyclin T1 and pUL69 was confirmed in HCMV-infected cells (m.o.i. of 1 or 2) in the absence or presence of roscovitine (10 μM). (c) After precipitation of cyclin T1 from cell lysates at 72 h p.i., immunoprecipitates were studied for pUL69 and CDK9. Staining of IE1 was used as specificity control. pAb g, Goat polyclonal antibody. (d) Expression of proteins of interest was monitored by WB analysis. (e) The yeast two-hybrid system was used to narrow down the protein domains responsible for the interactions identified by CoIP analysis. Signals obtained by staining of yeast colonies in a filter lift assay were categorized as strongly positive (+), intermediate or weakly positive (+) and negative (−). Constructs pVA3 and pTD1 expressing p53 and simian virus 40 large T antigen (SV40-T) were used as positive controls (Clontech).
specific antibodies were used to detect RNAPII in its differentially phosphorylated forms of the CTD. The putative co-localization of RNAPII with viral nuclear compartments in HCMV-infected cells was analysed by confocal laser-scanning microscopy. When using an antibody recognizing both the unphosphorylated and phosphorylated forms of CTD of RNAPII, co-localization with virus replication centres was detected (mAb-RNAPII; Fig. 4a, panels e–h). Even more strikingly, a distinct co-localization of RNAPII with pUL69 in inhibitor-induced speckled aggregates could be demonstrated (Fig. 4a, panels j–m and o–r). The inset images show that both proteins were found throughout these structures within replication centres (Fig. 4a, panel i), whilst in speckled aggregates the proteins co-localized mainly at their periphery (Fig. 4a, panels n and s). When specifically detecting the unphosphorylated form, a strong signal was found within virus replication centres (mAb-RNAPII-0; Fig. 4b, panels e–h), but association with speckled aggregates could only be detected to a minor extent (Fig. 4b, panels i–p). Further investigation of the localization of the phosphorylated forms did not clearly reveal distinct and reliable staining patterns but showed variability between separate experiments (mAb-RNAPII-5P and mAb-RNAPII-2P; data not shown). Therefore, the association of phosphorylated RNAPII subforms within speckled aggregates could not be safely defined by this experimental setting. However, the present data argue for co-localization of RNAPII with virus replication centres at late times of infection, and, as shown with the non-phospho-specific mAb-RNAPII, an association of RNAPII with CDK inhibitor-induced aggregates could also be demonstrated.

**pUL69 represents the defining component of nuclear speckled aggregates**

To address the question of which of the associated proteins might determine their recruitment to CDK inhibitor-induced speckled aggregates, we performed infection experiments with a bacterial artificial chromosome (BAC)-derived recombinant of HCMV AD169 lacking a functional UL69 gene (HB5AUL69). This virus does not express pUL69 (Fig. 5c) and develops a replication cycle that is inefficient and retarded in the kinetics of viral gene expression (R. Müller, S. Feichtinger & T. Stamminger, unpublished data). Despite these defects, HB5AUL69 formed normal virus replication centres in the nuclei of infected fibroblasts, as characterized by the specific organization of viral DNA processivity factor pUL44, used here as a marker protein (Fig. 5a, b, panels e–h). In addition, in the presence of roscovitine, the formation of virus replication centres appeared mostly unaffected (Fig. 5a, b, panels i–l). As a control, a BAC-derived recombinant HCMV lacking UL27 was analysed in parallel (TB40/EUL27; Le et al., 2008), showing an unaltered speckled aggregation of pUL69 comparable to HCMV AD169 (data not shown). As an important finding, however, HB5AUL69-infected cells were totally devoid of roscovitine-derived speckled aggregates. Neither CDK9 nor cyclin T1 was detectable in the location of any speckled structures in the late phase of virus replication (Fig. 5a, b, panel j). These findings strongly argue for pUL69 as the defining component of nuclear aggregates upon inhibition of CDK activity. Interestingly, in the case of HCMV HB5AUL69, cyclin T1 and CDK9 were detectable within replication centres even in the absence of pUL69 (Fig. 5a, b, panels f and j). Thus, although pUL69 appears to be the key component for the formation of speckled aggregates, another as yet undefined viral protein seems to be required for the recruitment of cyclin T1 and CDK9 to nuclear replication centres.

**Conclusions**

This study provides the first evidence for (i) the direct interaction between pUL69 and cyclin T1 in HCMV-infected fibroblasts, (ii) the specific role of CDK9 activity for the correct nuclear localization of viral and cellular proteins, (iii) the initial supposition that inhibition of CDK9 may be sufficient to induce a speckled aggregation of the pUL69–cyclin T1–CDK9 complex and to block virus replication in fibroblasts, (iv) the association of further proteins such as RNAPII with these aggregates and (v) the importance of pUL69 for cyclinT1–CDK9 interaction as the defining component of CDK inhibitor-induced aggregates.

In this work, we show direct binding of cyclin T1 to pUL69 in transfected 293T cells and in HCMV-infected fibroblasts. The fact that cyclin T1 and not CDK9 acts as the binding partner of pUL69 is in line with several findings of CDK9/ cyclin T1-associated proteins. Brd4 recruits CDK9 via its binding to cyclin T1 to the paused RNAPII at acetylated promoter regions to enhance transcription elongation (Yang et al., 2008). CDK9 localization seems generally dependent on cyclin T1-binding in both uninfected and HCMV-infected fibroblasts (Kapasi et al., 2009; Napolitano et al., 2002). Along with previous results (Rechter et al., 2009), the identification of a cyclin T1–pUL69 interaction indicates recruitment of CDK9 to pUL69 via cyclin T1 in order to enable pUL69 phosphorylation and, consequently, the modulation of pUL69 activity.

Our studies with roscovitine and other CDK inhibitors on the localization pattern of viral and cellular nuclear proteins supported the idea of aggregation of a selective panel of proteins in the observed speckled nuclear structures. The specificity of proteins studied indicated that pUL69, CDK9, cyclin T1 and RNAPII could be identified as constant components in speckled aggregates, whereas other proteins, such as HDAC2, CDK7, cyclin B1, SC-35, pp65, pp71, IE2p86, pUL26, pUL44, pUL50, pUL84, pUL97 and pUL112–113, were found not to be associated. These proteins either remained in their typical localization or were recruited to virus replication centres but were never detectable in speckled aggregates (Rechter et al., 2009; data not shown).

The phenomenon of pUL69 speckled aggregation induced by roscovitine seems not to be confined to HCMV.
laboratory strain AD169 but has also been detected for AD169-derived virus variants carrying ganciclovir/cidofovir resistance and for other viral strains (e.g. Towne; Rechter et al., 2009). Here, we additionally analysed clinical HCMV isolates (29A and 5B) and a novel multidrug-resistant HCMV (A834P; Scott et al., 2007). All viruses showed pronounced aggregation of pUL69, which was very similar to the pattern described for the AD169 reference strain (data not shown). Thus, the known differences in the coding capacity of these viruses do not provide functions that render pUL69 independent from roscovitine- or R22-induced aggregation. Several lines of evidence indicate that CDK inhibitor-induced speckled aggregates may represent nuclear structures in which the normal function of cellular RNAPII in relation to pUL69. HFFs were infected with AD169 (m.o.i. of 1) and cultured with roscovitine or R22 from 24 h.p.i. At 72 h, cells were used for immunofluorescence staining for the detection of pUL69 and different forms of the CTD of RNAPII. Detection antibodies were used that either recognized the unphosphorylated as well as the phosphorylated forms (a) or selectively recognized the unphosphorylated form of the CTD of RNAPII (b). Inset images show enlargements of representative areas of the replication centres or speckled aggregates.

**Fig. 4.** Distribution pattern of cellular RNAPII in relation to pUL69. HFFs were infected with AD169 (m.o.i. of 1) and cultured with roscovitine or R22 from 24 h.p.i. At 72 h, cells were used for immunofluorescence staining for the detection of pUL69 and different forms of the CTD of RNAPII. Detection antibodies were used that either recognized the unphosphorylated as well as the phosphorylated forms (a) or selectively recognized the unphosphorylated form of the CTD of RNAPII (b). Inset images show enlargements of representative areas of the replication centres or speckled aggregates.
associated proteins is suppressed. This suppression may add to the strong inhibitory effect that CDK inhibitors exert on HCMV replication. CDK-specific regulation can be manifested at several, so far poorly characterized regulatory steps of the HCMV replication cycle, such as protein phosphorylation, modulation of protein activity, protein

**Fig. 5.** Localization of CDK9 and cyclin T1 (cct1) in cells infected with a recombinant UL69-deleted virus (HCMV HB5ΔUL69). HFFs were infected with HB5ΔUL69 for 9 days (until the onset of visually detectable cytopathic effects induced by the decelerated virus replication), followed by roscovitine treatment for 48 h. Localization of CDK9 (a) and cyclin T1 (b) was visualized using specific antibodies. In addition, viral pUL44 was stained with a pUL44-specific antibody as a marker for nuclear replication centres. (c) A control staining of HB5ΔUL69-infected cells with a specific antibody confirmed the absence of pUL69 expression.
interactions and transport pathways. The data presented in this study support the evolving concept that CDK9/cyclin T1 activity is highly associated with the regulatory functions of pUL69 and thus with the efficiency of HCMV replication. Further detailed aspects of the molecular mechanism of CDK9/cyclin T1–HCMV inter-regulation need to be elucidated in future studies.

METHODS

CDK inhibitors and reference compounds. Roscovitine was purchased from Calbiochem and CDK inhibitor R22 (aminopyrimidine) was provided by GPC Biotech AG. R22 is the product of a series of medicinal chemistry optimization cycles, based on hit compounds identified in a high-throughput screening of purified recombinant CDK9/cyclin T1 complex against a kinase-biased screening collection. The characteristics of R22 indicate a high CDK9 potency, cellular CDK9 activity and selectivity within the kinase family. Moreover, several pharmacological properties, such as solubility, bioavailability and metabolic stability, appear very promising. Compounds were prepared in DMSO and aliquots were stored at −20 or at −80 °C (for periods longer than 3 months).

Selectivity of CDK inhibitor R22. For selectivity panels of R22, various cellular protein kinases were assayed for activity by a [33P]-ATP-based in vitro kinase assay (Herget et al., 2004; Mett et al., 2005). Assays were adequate when the Z prime value was >0.5. Reactions were performed in the linear range for reaction time and enzyme concentration as well as an ATP concentration according to the K_m of the respective enzyme for comparison of the inhibitory effects of the compounds, as described previously (Mett et al., 2005). In addition to the radioactive in vitro kinase assays, substrate phosphorylation was determined in an alternative assay format by an immobilized metal assay of phosphorylation (IMAP, Molecular Devices; Sportsman et al., 2004). IMAP technology is based on the covalent-coordinate, high-affinity interaction of trivalent metal-containing nanoparticles with phospho-groups linked to serines, threonines or tyrosines. In brief, fluorescence-labelled peptides were phosphorylated in a kinase reaction in a microtitre plate-based format. The reaction was stopped by addition of the IMAP reagent (Molecular Devices), which contained IMAP beads binding to phosphorylated substrate peptides, thereby leading to an increase in fluorescence polarization upon phosphorylation. Signals were detected by fluorescence polarization measurement in a Victor Wallac reader (Perkin Elmer).

Cell culture, HCMV infections and plasmid transfection. 293T cells were cultivated in Dulbecco’s modified Eagle’s medium containing 10% FBS. HFFs were cultivated in minimal essential medium containing 7.5% FBS. HCMV laboratory strain AD169 was propagated in HFFs. Titters of virus stocks were determined using standard plaque titration. HFFs (2.5 × 10^5 cells per well in a six-well plate) were infected as reported previously (Machera et al., 2000). Transfection of 293T cells was performed using polyethylenimine reagent (Sigma) as described previously (Schregel et al., 2007).

Generation of recombinant UL69-deleted HCMV HB5.AUL69. The recombinant AD169-derived HCMV HB5.AUL69 was generated, isolated and propagated as described previously (Tavalai et al., 2008). Briefly, BAC mutagenesis was performed via homologous recombination in Escherichia coli using a linear recombination cassette and the parental BAC pHBS (Borst et al., 1999). The recombination cassette consisted of a kanamycin resistance gene framed by 50 nt with homology to the 5’- and 3’-flanking regions of UL69. The respective fragment was generated by PCR amplification using the primer pair F-P1UL69-3 (5’-AACGGGATACAGGAGCACCATGG- CAGGCAACACCCCCCTCCTCCTTCTTGTGACTGGCTGAGCTG- CTTC-3’) and R-P4UL69-5 (5’-CTATATATACATGCGTGCCG- GAACGCTACTCTCTGAGGCGGCCTATCCCGGGGATCCG- TGACC-3’) together with plasmid pKD13 as template, which codes for the kanamycin resistance marker (Datsenko & Wanner, 2000). In the next step, homologous recombination in E. coli was performed as described previously (Tavalai et al., 2008). Thereafter, the structural integrity of the recombinant BAC was confirmed via restriction enzyme digestion and nucleotide sequence analysis of the recombined gene region. BAC DNA of positive recombinants was isolated using the Nucleobond AX 100 kit according to the manufacturer’s instructions (Machery Nagel). For the subsequent reconstitution of recombinant viruses, HBFFs were seeded into six-well dishes at a density of 3.5 × 10^5 cells per well and transfected with 1 μg of the respective BAC DNA together with an expression plasmid for pp71 using the transfection reagent FuGene HD according to the manufacturer’s protocol (Roche). One week after transfection, the cells were transferred into 25 cm² flasks and incubated until an almost complete cytopathic effect occurred so that the supernatant could be used to infect fresh HFF cultures for the preparation of virus stocks.

HCMV GFP-based antiviral assay. HFFs cultivated in 12-well plates were used for a GFP-based antiviral infection assay as described previously (Marschall et al., 2000). In brief, HFFs were infected with a recombinant GFP-expressing HCMV, AD169–GFP, at an m.o.i. of GFP of 0.25 (i.e. 25% GFP-forming infectious dose at 7 days p.i.). Infections were performed in duplicate. CDK inhibitors were added after virus adsorption, and at 7 days p.i. cells were lysed. Lyastes from each well were divided into two samples before processing and subjected to automated GFP quantification in a Victor 1240 Multilabel Counter (Perkin-Elmer Wallac).

Indirect immunofluorescence assay and confocal microscopy. HFFs were grown on coverslips in six-well plates (3 × 10^5 cells per well) and infected with AD169 at an m.o.i. of 1 or with HCMV HB5AUL69. For infection with HB5AUL69, HFFs were infected with 1 × 10^4 p.f.u. and incubated for 7 days until infection of cells was microscopically observable. CDK inhibitors were added to the culture medium at 24 h.p.i. for HCMV AD169 or at 7 days p.i. for HB5AUL69, followed by a change of the culture media including inhibitors every 24 h. Cells were fixed after 2 days of CDK inhibitor treatment using 4% paraformaldehyde (10 min, room temperature) and permeabilized using 0.2% Triton X-100 in PBS (20 min, 4 °C). Cells were incubated with the following primary antibodies for 60 min at 37 °C: mAb-UL69 (clone 69-66, kindly provided by W. Brit, University of Birmingham, AL, USA), mAb-UL44 (clone BS 510; kindly provided by B. Plachter, University of Mainz, Germany), mAb-CDK9 (clone D-7; Santa Cruz Biotechnology), mAb-RNAPII (clone CTD4H8; Covance), mAb-RNAPII-0 (clone 8WG16; Covance), mAb-RNAPII-5P (clone H14; Covance) and mAb- RNAPII-2P (clone H5; Covance); and polyclonal antibodies pAb-UL69 (Winkler et al., 1994), pAb-IE2 (anti-pHP178; Hofmann et al., 2000), pAb-UL97 (kindly provided by D. Michelis, University of Ulm, Germany), goat pAb-cycT1 (Santa Cruz Biotechnology) and pAb-cycB1 (Santa Cruz Biotechnology). Secondary antibodies used for triple staining in green (Alexa Fluor 488-conjugated antibodies; Molecular Probes), red (Alexa Fluor 555-conjugated antibodies; Molecular Probes), and far-red fluorescence (Alexa Fluor 647-conjugated antibodies) were incubated for 30 min at 37 °C (nuclear counterstaining with DAPI Vectashield mounting medium; Vector Laboratories). Immunofluorescence data were collected by confocal laser-scanning microscopy with a TCS SP5 microscope (Leica).

Co-immunoprecipitation assay. 293T cells were transfected with expression plasmids in 10 cm dishes via a polyethylenimine transfection.
tion technique as described previously (Schrexl et al., 2007) and lysed 2 days post-transfection. For interaction studies with proteins from infected cells, HCMV AD169-infected HFFs (m.o.i. of 1) were treated with CDK inhibitors (as described for indirect immunofluorescence) and lysed at 72 h p.i. Both transfected 293T cells and HCMV-infected HFFs were lysed in 500 μl CoIP buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, 2 μg aprotinin ml⁻¹, 2 μg leupeptin ml⁻¹ and 2 μg pepstatin ml⁻¹] and used for CoIP with 1–5 μl antibody as indicated for 2 h at 4 °C under rotation in the presence of protein A–Sepharose beads (2.5 mg; Amersharm Pharmacia Biotech). The precipitates were then pelleted in a microfuge (10 000 r.p.m., 1 min) and washed five times (800 g each) and mediates cellular motility. Proc Natl Acad Sci U S A 106, 22369–22374.

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Molecular targets for human cytomegalovirus infection specifically alters the levels and localization of the RNA polymerase II carboxyl-terminal domain kinases cdk9 and cdk7 at the viral transcriptosome. (Marschall, M. & Stamminger, T. (2009).


