Profiling of the kinome of cytomegalovirus-infected cells reveals the functional importance of host kinases Aurora A, ABL and AMPK

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ABSTRACT

Human cytomegalovirus infection can lead to life-threatening clinical manifestations particularly in the immunocompromised host. Current therapy options face severe limitations leading to a continued search for alternative drug candidates. Viral replication is dependent on a balanced interaction between viral and cellular proteins. Especially protein kinases are important regulators of virus-host interaction indicated by remarkable kinome alterations induced upon HCMV infection. Here we report a novel approach of kinome profiling with an outcome that suggests an important role of specific cellular protein kinases, such as AMPK, ABL2 and Aurora A. Inhibition of AMPK and ABL kinases showed a significant reduction, whereas inhibition of Aurora A kinase led to a slight activation of HCMV replication, as measured in a GFP reporter-based replication assay. Furthermore, analysis of the mode of antiviral action suggested a substantial benefit for the efficiency of viral replication at the immediate early (AMPK) or early–late (ABL) phases of HCMV gene expression. In contrast, inhibition of Aurora A kinase promoted an enhancement of viral early–late gene expression, suggesting a putative role of Aurora A signaling in host defense. Thus, the combined data provide new information on host cell kinases involved in viral replication and uncovered potential targets for future antiviral strategies.

1. Introduction

Human cytomegalovirus (HCMV) is a worldwide distributed pathogen with seropositivity rates in the adult population ranging between 40% and 90% (Mocarski et al., 2007). In immunocompromised persons, HCMV frequently causes systemic disease with clinical consequences including retinitis, pneumonitis or gastroenteritis. Furthermore, HCMV is the most common cause of congenital viral infections (Adler et al., 2007). Antiviral therapy is mainly based on inhibitors of viral DNA synthesis, such as ganciclovir (GCV), its prodrug valganciclovir (VGCV), foscarnet (FOS) and cidofovir (CDV; Lischka and Zimmermann, 2008). Concerning HCMV therapy, GCV or VGCV is presently considered as the gold standard. GCV treatment, however, is frequently accompanied by myelotoxicity or other adverse side effects. The success of therapy is additionally threatened by the selection of drug-resistant virus variants (Dropulic and Cohen, 2010). Recently, protein kinases involved in the regulation of HCMV replication have been considered as interesting novel drug targets (Andrei et al., 2009; Chou, 2008; Lischka and Zimmermann, 2008; Marschall et al., 2011; Marschall and Stamminger, 2009; Tandon and Mocarski, 2012).

Replication and spread of human HCMV is dependent on the balance of interactions between viral and cellular proteins. Protein kinases, known to act as key regulatory factors of virus–host interaction, display a number of functions during HCMV replication (Lee and Chen, 2010; Prichard, 2009). It is known that upon HCMV infection, the phosphorylation level of proteins is increased and several modulations on kinase-dependent signaling pathways are induced (Hertel et al., 2007; Yurochko, 2008). In order to validate protein kinases as new potential antiviral targets of HCMV replication, the kinome of HCMV-infected human cells was analyzed. Hereby, an upregulation and activation of cellular protein kinases, which were so far poorly characterized in the context of HCMV replication, could be detected. Thus, we present a novel approach of kinome profiling that indicates an important role of specific host kinases in HCMV replication (e.g., AMPK [5′-AMP-activated protein kinase], ABL and Aurora A). We provide new evidence that ABL tyrosine kinases exert an important regulatory function showing for the first time that ABL tyrosine kinases hold an important
regulatory function in the early–late phase of HCMV replication. Furthermore, Aurora A, a kinase with so far unknown importance for HCMV replication, apparently exerts a negative effect on the efficiency of HCMV replication. Thus, an improved knowledge about kinome alterations during HCMV infection and the identification of novel upregulated target-kinases provide a novel basis for potential antiviral strategies.

2. Material and methods

2.1. Cell culture and virus infection experiments

Primary human foreskin fibroblasts (HFFs) were cultivated under conditions described previously (Milbradt et al., 2010). For infection experiments, HCMV laboratory strain AD169 was used and for antiviral assays, infections were performed with recombinant AD169-GFP as described before (Marschall et al., 2000).

2.2. Chemical reagents

Ganciclovir (2-[(2-amino-6-hydroxy-purin-9-yl)-methoxy]-propan-1,3-diol, Sigma–Aldrich), compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]-pyrimidine, Sigma–Aldrich), SC78566 (1-(6-(6-methoxy-7-(3-(4-methylpiperazin-1-yl)propoxy)quinazolin-4-ylamino)benzo[d]thiazol-2-yl)-3-(thiophen-2-yl)urea, 4SC AG, Martinsried, Germany), SC78709 (1-(6-(6-methoxy-7-(3-(4-methylpiperazin-1-yl)propoxy)quinazolin-4-ylamino)benzo[d]thiazol-2-yl)-3-(thiophen-3-
urea, 4SC AG, Martinsried, Germany), Aurora A Inhibitor I (N-(2-chlorophenyl)-4-(4-(2-(4-ethylpiperazin-1-yl)-2-oxoethyl)phenylamino)-5-fluoropyrimidin-4-ylamino)benzamide, Selleck), MLN8054 (4-[[9-chloro-7-(2,6-difluorophenyl)-5H-pyrimido[5,4-d][2]benzazepin-2-yl]amino]benzoic acid, Selleck), imatinib (C29H31N7O, 4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]phenyl]-benzamide, Selleck), and staurosporine (C58H70O11N7S, 9S,10R,11R,13R-2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-11(methylamino)-9,13-epoxy-1H,9H-diindolo[1,2,3-ghi:3',2',1',i]pyrrolo[3,4-j]3',1',i]benzodiazonin-1-one, Calbiochem) were dissolved in DMSO and stored at −80 °C.

2.3. HCMV GFP-based antiviral assay

HCMV GFP-based replication assays were carried out at a duration of seven days (multi-round infection) as described before (Chou et al., 2011; Marschall et al., 2000; Rechter et al., 2006). All infections were performed in duplicate; GFP quantifications were performed in quadruplicate. Processing and evaluation of data was performed by the use of Excel (means and standard deviations) and online calculator software for statistics (http://www.physics.csbsju.edu/stats; unpaired Student’s t-test for P values).

2.4. Western blot analysis

For Western blot analyses, protein lysates from HCMV-infected HFFs (single-round of 12–72 h or multi-round infections of seven days) were subjected to standard SDS–PAGE followed by a transfer to nitrocellulose membranes as described previously (Thomas et al., 2009; Webel et al., 2011). Immunostaining was performed with a panel of monoclonal antibodies (see legends to Figs. 3 and 6–8).

2.5. Cell proliferation assay

HFFs were cultured in minimal essential medium lacking phenol red indicator (MEM51200, Invitrogen), supplemented with gentamycin, glutamine and 7.5% FCS, seeded in a 96-well plate (5000 cells/well) and treated with different concentrations of inhibitors or with the control staurosporine (STP) at a concentration of 1.1 μM. Inhibitor treatment was performed in duplicate. Three days after addition, cell proliferation was assayed by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) in accordance to the manufacturer’s instructions. Processing and evaluation of data was performed by the use of Excel (means and standard errors).

Fig. 2. Identification of kinases upregulated in a HCMV-specific manner. (A) A section of the mass spectrum from 582.96 to 588.71 m/z (mass-to-charge) exemplarily shows the SILAC peptide pair LFQQILSAVDYCHR of AMPK catalytic subunit alpha-2. Uninfected and HCMV-infected HFFs were grown and labeled as described for Fig. 1. The ratio of uninfected and infected samples is obtained from the relative abundance of the MS peaks at m/z of 583.96 (left arrow-marked peak, Mock) 587.30 m/z (right arrow-marked peak, Inf.) revealing a 2.43 fold upregulation of AMPK in infected cells in this single measurement. (B) The mean ratio (log 10 transformed) of quantified kinases from infected to uninfected HFFs (Inf./Mock) was determined from values obtained from two independent MS measurements (upregulation values >0). Data refer to the mean ratio of Inf./Mock values of 3 mg protein from two independent experiments with inverted SILAC labels. Hit kinases are labeled as white squares. (C) Ranking list of cellular kinases found to be highly upregulated in HCMV-infected HFFs. Mean values were calculated from both MS experiments in duplicate (except ⁄ where identification was only possible in a single MS experiment).
2.6. Kinase enrichment experiments

For stable isotope labeling by amino acids in cell culture (SILAC), HFFs were cultivated on either ‘light’ or ‘heavy’ SILAC MEM Alpha Modification medium (PAA) supplemented with gentamy- cin, glutamine and 10% dialysed FCS (GIBCO) containing either lysine-0 and arginine-0 or lysine-8 (15N2-15N4-lysine) and arginine-10 (15C6-15N4-arginine; Silantes, Munich, Germany; Ong et al., 2002; Sharma et al., 2009). 5.8 × 10^6 cells were seeded in 10 cm dishes and three days later, cells were split 1:3 and cultivated in SILAC media for further four days. Subsequently, cells were Mock-infected or infected with HCMV AD169-GFP at MOI of 0.5 and harvested three days later (single-round infection). Incorporation of labeled amino acids was monitored and found to exceed 95%. For kinase enrichment association experiments, HFFs were lysed in buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 0.25% Triton X-100, 3 mM MgCl2, 1 mM EDTA, 1 mM EGTA plus additives (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM Na3VO4, 10 mM Naf). After centrifugation, lysates were adjusted to equal protein concentrations and filtered through a 0.45 μm cellulose acetate filter. Kinase enrichment experiments were performed with a kinase-specific affinity matrix. The matrix was similar to the affinity beads described by Daub et al. (2008) with a mix of beads coated with different inhibitors. Instead of V116832, the structurally similar compound V113743 (Oppermann et al., 2009) was used. In brief, pooled lysates containing 6 mg of protein were incubated for 2.5 h at 4 °C with 60 μl of affinity beads. Subsequently, beads were washed with lysis buffer devoid of the additives and proteins were eluted by denaturation by incubation with buffer containing 1% LDS at 72 °C for 10 min (LDS sample loading buffer, Thermo Fischer Scientific, Waltham, MA, USA). Separation of proteins by electrophoresis and in-gel digests with trypsin were performed as described previously (Sharma et al., 2009).

3. Results

3.1. Human kinase enrichment detects an HCMV-dependent upregulation of host kinases

SILAC metabolically prepares cell populations for protein quantification by quantitative mass spectrometry (Ong and Mann, 2005). SILAC relies on metabolic incorporation of ‘light’ (lysine-0/arginine-0) or ‘heavy’ (lysine-8/arginine-10) variants of the amino acids lysine and arginine into proteins. This procedure generates two cell populations with differentially labeled proteomes but with identical biochemical properties. The origin of the proteins, however, can be distinguished by quantitative mass spectrometry due to a characteristic mass shift of the corresponding tryptic peptides (Fig. 1). In this approach, human foreskin fibroblasts (HFFs) were grown in SILAC media for labeling and used for HCMV infection (MOI of 0.5, three days). Protein kinases were enriched from cell lysates by an affinity matrix consisting of a panel of broad-spectrum kinase inhibitors. Enrichment was based on increased binding affinity of activated kinases and/or increased kinase expression levels, so that activation as well as upregulation could be detected (but not distinguished from each other). Bound protein was eluted and subsequently analyzed by LC-MS/MS (Fig. 2; for methodical details see Fig. 1 and Fig. S1). In preliminary settings, an optimization of experimental conditions was performed, including SILAC-labeling efficiencies, viral MOI and time points of infection. Particularly, the incorporation of the isotope-labeled amino acids was monitored by MS measurement during the labeling process revealing an almost entire incorporation (data not shown). Two independent kinase enrichment experiments were performed with a switch of the SILAC labeling scheme, of which a total of 96 kinases could be identified (for full mass spectra see Fig. S1; identified kinases are listed in Table S1). Data obtained from the two experiments nicely correlated in quantitative and qualitative terms (mean deviation was calculated as 2.35%). A representative SILAC peptide pair of AMPK catalytic subunit alpha-2 is shown in Fig. 2A, revealing a striking upregulation. The vast majority of protein kinases detected were upregulated upon HCMV-infection (Fig. 2B). This massive upregulation appears in line with the known initial stimulatory impact of HCMV replication onto proliferation and signaling activity of the host cell (Hertel et al., 2007; Yurochko, 2008). Particularly, the expression levels of eight cellular kinases were found to be substantially increased in infected cells (Fig. 2B and C). Thus, an upregulation was found for CDK1 (cyclin-dependent kinase 1), CDK2 (cyclin-dependent kinase 2), AMPK, MST1 (mammalian Sterile 20-like kinase 1), MST2 (mammalian Sterile 20-like kinase 2), ULK3 (unc-51-like kinase 3), ABL2 (v-abl Abelson murine leukemia viral oncogene homolog 2) and Aurora A kinase (Fig. 2C). The latter six kinases have poorly been characterized concerning their role during HCMV infection and should be further analyzed.

3.2. Experimental confirmation of the MS-detected upregulation of Aurora A, ABL2, AMPK, ULK3, MST1 and MST2

In order to verify kinase upregulation detected by quantitative mass spectrometry in HCMV-infected cells, the extracts of SILAC-labeled cells were analyzed by SDS–PAGE and Western blot (Fig. 3A). In parallel, HFFs were grown independently in regular medium, infected with HCMV AD169-GFP at a MOI of 0.5, harvested and lysed three days post-infection (p.i.). Cell extracts were applied to affinity beads to enrich kinases for SDS–PAGE and Western blot analysis (Fig. 3B). Comparing cell lysates obtained from SILAC-labeled with those from unlabeled infected HFFs (Fig. 3A and B), a strong increase in levels of Aurora A with respect to

![Fig. 3](image-url)
uninfected cells was detected. This underlines our MS findings of an upregulation by factor of 6.2 during HCMV replication (Fig. 2C). The level of the phosphorylated form of Aurora A, p-Aurora A (pT288, representing activated kinase) was found to be increased in infected cells. Notably, p-Aurora A was enriched to a lesser extent by affinity beads than total Aurora A (Fig. 3B). This might indicate that the pT288-phosphorylated form of Aurora A is poorly bound by the beads-associated inhibitors. It has been reported that the site-specific phosphorylation of the activation segment of the catalytic lobe of Aurora A is important for its kinase activity (Littlepage et al., 2002). Thus, a potential mechanism explaining our finding might be a reduced binding affinity based on a phosphorylation-dependent conformational change of Aurora A. This is in accordance with findings for another kinase, i.e. AMPK (see below) pointing to a phosphorylation-modulated binding affinity to inhibitors.

For ABL2, an upregulation as well as a modification in protein size could be determined in HCMV-infected SILAC-labeled cells (Fig. 3A). This might be due to the induction of faster migrating forms of ABL2 during HCMV replication. In contrast, protein samples after kinase enrichment solely contained ABL2 in its faster migrating, infection-specific form. Multiple alternatively spliced transcript variants encoding different protein isoforms have been found (Santamaria et al., 2010) which is compatible with our findings. We hypothesize, that the faster migrating species of ABL2 are most active in binding to the affinity beads suggesting a highly active state of the kinase. It should be stressed, however, that we could not distinguish between an activation-based or high-level expression-based enrichment of kinase affinity.

For AMPK, no HCMV-specific change in protein levels could be observed in SILAC-labeled cells (Fig 3A). Of note, however, kinase enrichment yielded increased amounts of AMPK in the eluted fraction of both, infected and uninfected samples (Fig. 3B, lanes 3–4). Interestingly, the phosphorylated fraction of AMPK, p-AMPK (pT172), was found heavily increased specifically in the infected samples subjected to kinase enrichment (Fig. 3B, lane 4). In the absence of kinase enrichment, no marked upregulation of p-AMPK was detectable, neither under SILAC conditions nor under normal growth conditions (Fig. 3A and B).

ULK3 and MST1/2 were found to be slightly upregulated in infected cells (Fig 3A and B). The difference between uninfected and infected cells became more obvious after kinase enrichment suggesting some degree of enhancement of ULK3 and MST1/2 during HCMV infection. As a control, β-actin staining was performed indicating an enrichment specific for protein kinases (Fig. 3B, lanes 1–2, compared to 3–4 lacking β-actin).

Fig. 4. MOI-dependent impact of HCMV infection on selected host kinases. HFFs were infected with HCMV AD169 at different MOIs (0.1, 0.25, 0.5, 1 or 3; see staining of IE1p72 as an infection control) or remained uninfected as a control (Mock). Cells were harvested 72 h.p.i. and the cell lysates were subjected to SDS-PAGE and Western blot analysis. Detection of specific proteins was performed as described for Fig. 3, antibody MAb-IE1p72 (63-27) was kindly provided by Britt (Univ. of Alabama, Birmingham, USA).

Fig. 5. Measurement of putative antiproliferative effects of AMPK, Aurora A and ABL inhibitors. HFFs were treated for three days with indicated concentrations of the kinase inhibitors compound C (AMPK), SC78566 (Aurora A, broad-spectrum), SC78709 (Aurora A, broad-spectrum), Aurora A Inhibitor I (Aurora A), MLN8054 (Aurora A) or imatinib (BCR-ABL, ABL1, ABL2, c-kit and PDGF-R), with the control staurosporine (STP; broad-spectrum) or left untreated. The release of NADPH/NADH was indirectly measured by their ability to reduce MTS tetrazolium (inner salt) to water-soluble formazan which can be quantified by its absorption at 490 nm. As a further control, absorption of medium without cells was measured (Medium only). Mean values of the controls (STP; Medium only) were obtained from measurements in quadruplicate and are depicted as straight bottom lines (standard error of each is 1%, not graphically displayed). Other mean values and appropriate standard error bars were obtained from measurements in duplicate.
In order to analyze kinase expression patterns more precisely in the context of various HCMV multiplicities, HFFs were infected with HCMV AD169 at MOIs from 0.1 to 3 (Fig. 4, lanes 2–6). A striking upregulation was detectable for ULK3, Aurora A and p-Aurora, whereas the expression of MST1/2 and AMPK was only slightly increased during infection (MOI-dependent increases in signal intensities were confirmed by densitometric quantification). Notably, all detectable changes in the expression levels occurred in a context of viral gene expression. (A) Compounds imatinib (BCR-ABL, ABL1, ABL2, c-kit and PDGF-R) and MLN8054 (Aurora A) were analyzed by a HCMV GFP-based replication system in which HFFs were infected with HCMV AD169-GFP at MOI of 0.01 (or remained uninfected as a control [Mock]). Antiviral compounds (including control GCV, 10 µM) were added in different concentrations (1.1 µM, 3.3 µM or 10 µM) immediately after infection. The cells were lysed seven days p.i. and GFP counts were quantified by automated fluorometry. Mean values of the controls (GCV; Mock) are depicted as straight bottom lines (standard error of GCV values is 1% and of Mock values 0%, not graphically displayed). (B) Mean values (n = 4) and appropriate percentages of GFP counts of different concentrations were used to calculate statistical significance by applying unpaired Student’s t-test. A P value <0.05 was considered as significant. (C) The IC50 value was calculated from data obtained from the HCMV GFP-based replication system (n = 4). Microscopically evaluated cytotoxicity was determined six days post-treatment at the indicated concentrations. None, no alteration of cell morphology or cytotoxicity; */C3, slight alteration of cell morphology; **, more pronounced alteration (in the absence of cell death). (D) HFFs were infected with HCMV AD169 at MOI of 0.3 (or remained uninfected as a control [Mock]). Kinase inhibitors, reference compound GCV (10 µM) or DMSO alone (Inf.) were added immediately after infection at the indicated concentrations. Cells were harvested 12 h, 24 h, 48 h and 72 h p.i. to perform SDS–PAGE and Western blot analysis. Detection of specific proteins was carried out with the following antibodies: MAb-IE1p72 (63–27), MAb-pp28 (41–18; both kindly provided by Britt, Univ. of Alabama, Birmingham, USA), MAb-UL44 (BS510; kindly provided by Plachter, Univ. Mainz, Germany; Becke et al., 2010) and MAb-β-actin (AC-15, Sigma).
MOI-dependent manner. For ABL2, the HCMV-specific shift to a lower migrating form could be identified comparable to results reported in Fig. 3. Combined, HCMV alters expression levels of numerous host kinases, so that for Aurora A, ABL2, AMPK, ULK3, MST1 and MST2 a potential role during HCMV replication is suggestive.

3.3. Inhibition of AMPK and ABL exerts a negative effect, while inhibition of Aurora A exerts a positive effect on HCMV replication

To verify the observed changes in kinase abundance upon HCMV infection, an ABL inhibitor (imatinib), two selective Aurora A inhibitors (MLN8054 and Aurora A Inhibitor I), two broad-spectrum kinase inhibitors with proven activity against Aurora A (SC78566, SC78709) and an AMPK inhibitor (compound C) were investigated in the context of HCMV infection. Interestingly, we detected different effects for the selective versus broad inhibitors of Aurora A.

Imatinib has been developed for cancer treatment, it selectively inhibits BCR-ABL, ABL1, and ABL2, as well as c-kit and platelet derived growth factor receptor (PDGF-R), but no other known tyrosine kinases (Antonescu, 2011; Buchdunger et al., 1996; Salah et al., 2011).

MLN8054 and Aurora A Inhibitor I are potent and selective inhibitors of Aurora A kinase. The selectivity of both inhibitors has been determined in smaller scales (data provided by the manufacturer), stating a high selectivity for Aurora A over Aurora B and Aurora C. Moreover, in case of MLN8054 a more detailed selectivity panel was published including a screen comprising 226 kinases (Manfredi et al, 2007). Furthermore, Aliagas-Martin et al. (2009) described an exceptional selectivity of Aurora A Inhibitor I (referred to as Inhibitor 10) in that no indications were given for inhibition of Aurora B and CDK2.

SC78566 and SC78709 show a broad inhibitory activity against a number of kinases including Aurora A. These compounds were

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**Table 1:**

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<th>Inhibitor</th>
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<th>1.1 µM P value</th>
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**Table 2:**

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<td>Compound C</td>
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**Fig. 7.** Influence of SC78566, SC78709 and compound C on HCMV replication and individual stages of viral gene expression. (A) Compounds SC78566 (Aurora A, broad-spectrum), SC78709 (Aurora A, broad-spectrum) and compound C (AMPK) were used for infection and quantification of GFP fluorometry under conditions described for Fig. 6A. Mean values of the controls (GCV; Mock) are depicted as straight bottom lines (standard error of GCV values is 1% and of Mock values 0%, not graphically displayed). (B), (C) and (D) Experimental procedures and evaluations were identical to those described in Fig. 6, antibody MAb-gB (27-287) was kindly provided by Mach (Univ. Erlangen-Nuremberg, Germany).
developed by 4SC AG (Martinsried, Germany) and proved to potentially inhibit the in vitro activity of TRK-A (neurotrophic tyrosine kinase receptor type 1), Aurora A, Aurora B, SAK (Snk/P1k-akinase), TIE2, TYRO3 and FLT-3 (Fms-like tyrosine kinase 3) in a picomolar range (data not shown).

Compound C acts as a specific inhibitor of AMPK and alters AMPK phosphorylation. AMPK is activated through binding of AMP and phosphorylation of AMPK alpha residue T172 whereas ATP leads to an inhibition of AMPK activity (Davies et al., 1995).

Firstly, proliferation assays were carried out in order to monitor putative antiproliferative effects of the inhibitors (Fig. 5). We limited the duration of proliferation measurements to three days, a period in which HFFs were grown to confluence. HFFs were treated with indicated concentrations of compound C, SC78566, SC78709, Aurora A Inhibitor I, MLN8054, imatinib or with the broad-spectrum kinase inhibitor staurosperine (STP, 1.1 M) known to arrest cells at the G1 checkpoint (McGahren-Murray et al., 2006). Values obtained from STP-treated cells indicated a total inhibition of cell proliferation (Fig. 5). Aurora A Inhibitor I showed a strong and concentration-dependent antiproliferative effect with a maximum of 81% at a concentration of 10 M. In contrast, proliferation of cells treated with MLN8054 was unaffected. Treatment with SC78566, SC78709, compound C or imatinib partially reduced proliferation in a dose-dependent manner to a maximum of approximately 50% at concentrations of 10 M. This may indicate an intermediate influence of the compounds onto cellular proliferation activity and we cannot exclude partial cytotoxicity at least under high concentrations and long-term treatment. It should be stressed, however, that microscopic inspection of cells and analysis of β-actin levels on Western blots did not indicate any sign of cytotoxicity even after seven days of treatment with these compounds (see Figs. 6–8). In general, mechanisms of antiviral activity can be linked to antiproliferative effects. However, since HCMV itself induces an early cell cycle arrest, this mechanism is unlikely to be fully responsible for the described antiviral drug activities.

Next, imatinib, MLN8054, SC78566, SC78709 and compound C were analyzed in an antiviral assay determining the replication efficiency of HCMV AD169-GFP in HFFs (Marshall et al., 2000; anti-HCMV screening assays were conducted at low MOI appropriately adjusted to quantitate the potency of antiviral compounds, since earlier experimentation demonstrated a less pronounced drug efficacy at higher MOI). Importantly, imatinib, SC78566, SC78709, compound C as well as the reference drug GCV exerted an anti-HCMV activity clearly distinct from cytotoxic effects as evaluated by light and fluorescence microscopy (Figs. 6A–C and 7A–C). Notably, the inhibitors imatinib, SC78566, SC78709 and compound C significantly reduced viral replication in a dose-dependent manner, with IC50s of 6.85 ± 0.42 μM, 2.72 ± 0.31 μM, 4.81 ± 1.88 μM, and 0.88 ± 0.01 μM, respectively (for P values see Figs. 6B and 7B). This anti-cytomegaloviral efficacy demonstrates the importance of ABL kinases and AMPK for an efficient HCMV replication in primary human fibroblasts. Unexpectedly, treatment with the Aurora A inhibitor MLN8054 showed signs of increased replication compared to the untreated control (for P values see Fig. 6B; the Aurora A Inhibitor I induced a slight, but not statistically significant increase of HCMV replication at a concentration of 1.1 M or lower; data not shown). The effect of MLN8054 suggests a negative functional role of Aurora A during HCMV infection which might be connected with antiviral host defense. Interestingly, the use of non-selective inhibitors of Aurora A kinase (SC78566, SC78709) did not show increase in HCMV replication, but in contrast substantially decreased replication efficiency. This may be explained by the concurrent inhibition of virus-supportive cellular kinases through these compounds. Furthermore, we addressed the question if dual treatment with Aurora A inhibitor and GCV leads to an antagonistic effect. HCMV GFP-based replication assays were conducted in the presence of GCV, MLN8054 and combinations of MLN8054 and GCV. In parallel, standard plaque reduction assays were performed. In the presence of MLN8054, the anti-HCMV activity of GCV was abrogated to only a slight but significant level. Replication efficiency was 37% (0.12 μM GCV alone) compared to 49% (0.12 μM GCV plus 0.12 μM MLN8054), and 35% (0.37 μM GCV alone) compared to 46% (0.37 μM GCV plus 0.37 μM MLN8054; Fig. 5). In general, these data demonstrate a functional importance of host kinases Aurora A, ABL kinases and AMPK during HCMV replication.

3.4. Differential impact of host kinase inhibitors on HCMV replication: immediate early block (AMPK), early–late block (ABL) or early–late upregulation (Aurora A)

For further evaluation of the antiviral mode of activity, we analyzed the effect of kinase inhibitors during the course of HCMV replication on the expression levels of viral immediate early (IE1p72), early (pUL44), early–late (gB) and true late (pp28) proteins. For this purpose, HFFs were infected with HCMV AD169 at a MOI of 0.3 and treated with the kinase inhibitors immediately after infection. Imatinib effected a reduction of expression level of pUL44 at a concentration of 10 μM, 48 h and 72 h p.i. (Fig. 6D). More striking became the inhibitory effect of imatinib at 72 h p.i., when a strong reduction of pp28 levels was demonstrated compared to the DMSO control (Fig. 6D, lanes 7 and 9). Moreover, SC78709 and compound C caused a substantial delay of accumulation of each analyzed viral protein at concentrations of 10 μM (Fig. 7D, lanes 4 and 7) whereas the expression was not or only marginally affected at concentrations of 3.3 or 1.1 M. In contrast, inhibition of Aurora A (MLN8054) did not reduce but accelerated gene expression of pUL44 48 h p.i. at concentrations of 3.3 and 1.1 μM (Fig. 6D, lanes 2, 4 and 5). As a control, the expression of the unrelated protein (β-actin) was not influenced by the inhibitors (Figs. 6D and 7D). Notably, the pattern of inhibition observed for SC78709 and compound C differed from the effect of GCV, i.e. 12 h p.i., IE1p72 levels were decreased in the presence of these kinase inhibitors but not for GCV. Thus, inhibition of host kinases by SC78709 and compound C blocked viral protein expression already at the immediate early stage of the replication cycle (Fig. 7D). In contrast, the pattern of inhibition of ABL tyrosine kinases (imatinib) was very similar to...
that obtained for GCV (Fig. 6D, lanes 8 and 9; note a transient reduction of IEp72 levels 12 h p.i. which was reversed at later time points). Therefore, we conclude an imatinib-mediated early-late block of viral gene expression. To illustrate the HCMV-activating effect observed with MLN8054 at a MOI of 0.3, we repeated this experiment at a lower MOI (0.01) using Aurora A Inhibitor I (Fig. 8; duration of HCMV infection seven days). Under these conditions, again an enhanced synthesis of viral proteins was detected at concentrations of 0.37 or 1.1 μM (compared to the DMSO control, lane 2). Upregulation was demonstrated for viral IEp72, pUL44 and pp28 proteins (Fig. 8, lanes 5 and 6). Taken together, Aurora A exerts an enhancement of viral gene expression while AMPK and ABL contribute to the efficiency of viral replication at the immediate early and early-late phases, respectively.

4. Discussion

HCMV replication is dependent on the virus specific alteration of host cell pathways such as cell cycle control, transcription and nucleocytoplasmic translocation. Infection experiments were based on the HCMV laboratory strain AD169 utilizing its high replication efficiency in primary fibroblasts (possessing highest permissiveness compared to other cells). It should be mentioned that AD169 strain has been extensively cultured in fibroblasts and many genetic alterations have accumulated during the course of these passages. These genetic changes contribute to attenuation and affect the ability to replicate and disseminate in vivo. Thus, infection experiments with AD169-infected HFFs serve as a valuable model, although it may have limitations for the in vivo situation. Multiplicities of infection were mainly adjusted to an optimal detection of antiviral efficacy (antiviral replication assays performed at low MOI) or a high sensitivity of protein identification (Western blot analyses and kinase enrichment experiments performed at higher MOIs; with exception of Fig. 8 for the reason of MOI comparison with Fig. 6D). Although a uniform MOI for all experiments would have been desirable, this variation in MOI appeared to be necessary to optimally evaluate the different methodological settings used. Hence, the use of adjusted MOIs led to an important interpretation of our findings, in that functions of protein kinases during HCMV replication were not restricted to defined MOIs.

As a major finding, our experiments broadly identified influences of HCMV infection on the cellular kinase. Among the HCMV-mediated upregulation of host kinases found in the present study, Aurora A could be identified as a negative regulator of HCMV replication. This corresponds to the recently published result of a siRNA screening analysis of the human kinase during HCMV replication. The siRNA-mediated knockdown of Aurora A was found to increase virus yields, thus considered as a potential candidate restricting virus replication (Terry et al., 2012). Aurora A is a multifunctional serine/threonine-specific kinase required for the control of various cellular processes (Giet and Prigent, 1999; Kollareddy et al., 2008; Marumoto et al., 2005). Activation of Aurora A occurs downstream of CDK1/cyclin B (Bischoff et al., 1998; Marumoto et al., 2002) and it could be demonstrated, that CDK1 activity is required for activation of Aurora A during G2/M phase transition of human cells (van Horn et al., 2010). Elevated level of Aurora A during HCMV replication may indicate a functional link of Aurora A to a modulation of the cell cycle that has negative effects on HCMV replication and, moreover, might play a role in cellular antiviral defense.

Furthermore, our findings demonstrate an HCMV-dependent modulation of AMPK. In particular, levels of the phosphorylated form of AMPK (at alpha residue T172; p-AMPK, Fig. 3B) were found to be enhanced in HCMV-infected cells. The importance of AMPK for efficient HCMV replication was confirmed by the detection of an antiviral effect of an AMPK-inhibiting compound (compound C; Davies et al., 1995). As a key sensor of metabolic activities, the main function of AMPK is to regulate the energy status of the cell (Hardie and Sakamoto, 2006). In order to protect cells from stress that result in a drop of ATP, AMPK is able to switch off ATP-consuming biosynthetic pathways. Activation of AMPK is mediated by high levels of AMP and low levels of ATP through a mechanism that involves allosteric regulation, inhibition of dephosphorylation and promotion of phosphorylation mainly at the key regulatory site T172 (Fig. 3B). Recent reports showed that HCMV infection can lead to an activation of AMPK. This may contribute to cellular glycolytic activity and an efficient viral replication (McArdle et al., 2012; Terry et al., 2012). Next, ABL protein kinases (ABL1 and paralog ABL2) were found relevant for HCMV, since they play a role in several cellular key processes, such as cell proliferation and survival (Pendergast, 2002; Sirvent et al., 2008). ABL kinases are activated by stimulation of growth factor receptor tyrosine kinases such as the epidermal growth factor receptor (EGF-R) and PDGF-R (Plattner et al., 2004, 1999). It is known, that activation of ABL kinases occurs also in response to the entry of microbial pathogens into mammalian cells (Backert et al., 2008; Burton et al., 2003; Tegtmeyer and Backert, 2011). ABL2 kinase signaling can be hijacked by these pathogens to reorganize the host actin cytoskeleton for the benefit of intracellular movement and release from the host cell. In addition, ABL family kinases play an important role for viral replication, as demonstrated for mouse polyomavirus and human BK virus (Swimm et al., 2010). The latter report described that ABL1 and ABL2 act redundantly to support virus infection. Initial experimental evidence already pointed to a possible importance of ABL1 for HCMV replication (Terry et al., 2012). In addition, our study identified MST1, MST2 and ULK3 as further candidate kinases supporting HCMV replication. For these protein kinases, no regulatory role during HCMV infection has been addressed so far.

5. Conclusions

Our data provide evidence that Aurora A, AMPK, ABL and CDKs are functionally integrated into HCMV replication. Inhibition of AMPK and ABL kinases exerted a negative effect, inhibition of Aurora A kinase a slightly positive effect on HCMV replication. In particular, our data point to an important new role of ABL tyrosine kinases during HCMV replication. As far as the mode of antiviral action of these kinase inhibitors is concerned, a replicative block was demonstrated at the immediate early (AMPK) or early–late (ABL) phases of HCMV gene expression. Interestingly, inhibition of Aurora A kinase promoted an enhancement of viral early–late gene expression. The regulatory mechanisms have to be elucidated in future studies. Thus, this comparative kinome analyses between HCMV-infected versus uninfected primary cells provides novel information on host cell determinants of viral replication. The identified cellular protein kinases represent potential targets for future antiviral strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.04.017.

References


