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The eIF4AIII RNA helicase is a critical determinant of human cytomegalovirus replication

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Abstract

Human cytomegalovirus (HCMV) was recently shown to encode a large number of spliced mRNAs. While the nuclear export of unspliced viral transcripts has been extensively studied, the role of host mRNA export factors in HCMV mRNA trafficking remains poorly defined. We found that the eIF4AIII RNA helicase, a component of the exon junction complex, was necessary for efficient virus replication. Depletion of eIF4AIII limited viral DNA accumulation, export of viral mRNAs from the nucleus, and the production of progeny virus. However eIF4AIII was dispensable for the association of viral transcripts with ribosomes. We found that pateamine A, a natural compound that inhibits both eIF4AI/II and eIF4AIII, has potent antiviral activity and inhibits HCMV replication throughout the virus lytic cycle. Our results demonstrate that eIF4AIII is required for efficient HCMV replication, and suggest that eIF4A family helicases may be a new class of targets for the development of host-directed antiviral therapeutics.

Keywords

Human herpesvirus; Human cytomegalovirus; Protein synthesis; mRNA export; Gene expression; eIF4AIII

Introduction

Post-transcriptional control of gene expression is a highly regulated process in mammalian cells. Following transcription primary transcripts undergo substantial remodeling in the nucleus. A multi-component complex termed the spliceosome recognizes and removes introns from primary mRNAs (Jurica and Moore, 2003). Concomitant with splicing, RNA binding proteins are deposited on the mRNA and signal that the mRNA is mature and ready for export to the cytoplasm. The mature, spliced mRNA then transits through the nuclear pore to the cytoplasm where it is translated into protein (Dreyfuss et al., 2002). Each step in this process requires a distinct complement of protein complexes and is regulated in response to environmental cues (Chang et al., 2013). The coordinated actions of the splicing machinery and subsequent RNA binding proteins ensure that only fully mature mRNAs are

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Appendix A. Supplementary material
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released to the cytoplasm for translation into protein (Wang and Burge, 2008; Parsyan et al., 2011).

Splicing was traditionally thought to have a limited role in expanding the complexity of herpesvirus proteomes. However, several recent studies have revealed extensive splicing of herpesvirus transcriptomes (Gatherer et al., 2011; Stern-Ginossar et al., 2012). Human cytomegalovirus (HCMV) encodes at least 132 spliced mRNAs (Gatherer et al., 2011), and many HCMV transcripts undergo alternative splicing. The widespread post-transcriptional processing of viral mRNAs likely accounts for part of the surprisingly complex HCMV proteome (Stern-Ginossar et al., 2012). In addition, these data suggest that host factors required for the transport of spliced mRNAs may play important roles in HCMV replication.

One host factor involved in the export of spliced mRNAs from the nucleus is the eIF4AIII RNA helicase. Interactions between eIF4AIII and the spliceosome result in eIF4AIII deposition on spliced mRNAs proximal to newly ligated exon junctions, although eIF4AIII is not an essential component of the spliceosome (Ferraiuolo et al., 2004; Shibuya et al., 2004; Shiimori et al., 2013). While eIF4AIII itself has no RNA binding specificity, two associated proteins, Y14 and magoh, coordinate eIF4AIII deposition adjacent to splice junctions (Bono and Gehring, 2011). Together with the barentsz protein, eIF4AIII, Y14, and magoh comprise the core proteins of the exon junction complex, or EJC (Palacios et al., 2004). After nucleation on splice junctions, the EJC core promotes the recruitment of additional proteins that facilitate nuclear export of spliced mRNAs (Bono and Gehring, 2011).

eIF4AIII also contributes to mRNA quality control and translation in the cytoplasm. Mature mRNAs are exported from the nucleus bound by the cap binding complex (CBC). In the cytoplasm, the CBC promotes the initial, or pioneer, round of translation during which bound EJCs are removed from the mRNA. Failure to remove EJCs targets the mRNA for degradation through the nonsense-mediated decay pathway (Alexandrov et al., 2012; Chang et al., 2007). In addition, the CBC recruits eIF4AIII to the 5′ end of the mRNA, where eIF4AIII helicase activity resolves secondary structure in the 5′ untranslated region (5′UTR) that otherwise impedes ribosome scanning. While the CBC facilitates the translation of some viral mRNAs (Sharma et al., 2012), the potential role of eIF4AIII in viral protein synthesis has not been determined.

In this study we found that the host eIF4AIII protein is required for efficient HCMV replication. Depletion of eIF4AIII decreased viral DNA replication, the expression of late transcripts, and limited the production of progeny virus. We found that depletion of eIF4AIII limits the cytoplasmic accumulation of HCMV mRNAs, suggesting a role for eIF4AIII in the nuclear export of viral transcripts. HCMV transcripts efficiently associate with polysomes in eIF4AIII-depleted cells, suggesting that eIF4AIII is not required for the translation of viral mRNAs. Based on the requirement for eIF4AIII for efficient virus replication and our previous work showing that inhibiting the related eIF4AI/II helicases decreased virus replication, we tested if pateamine A, a bioavailable natural compound that inhibits all three eIF4A family members, had antiviral activity against HCMV. We found that pateamine A potently inhibited HCMV replication during all stages of the virus lytic cycle at

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doses that did not affect the viability of uninfected cells. Together our results demonstrate that eIF4AIII is required for efficient HCMV replication, and suggest that eIF4A family helicases may be a new class of targets for the development of host-directed antiviral therapeutics.

Results

eIF4AIII is required for efficient HCMV replication

To determine if eIF4AIII is required for HCMV replication, we measured HCMV replication in eIF4AIII-depleted cells. Confluent fibroblasts were transduced with lentivirus expressing eIF4AIII-specific shRNAs (Fig. 1A), and the production of cell free virus in the supernatant was measured over a single round of viral replication. eIF4AIII depletion resulted in a greater than 150 fold defect in virus production at all times measured as compared to control cells expressing a scrambled shRNA with no predicted targets (Fig. 1B). Similar results were obtained in cells expressing a second eF4AIII-specific shRNA (Fig. S1). We conclude that eIF4AIII is required for efficient HCMV replication.

To determine where the block in virus replication occurred in eIF4AIII-depleted cells, we measured the expression of representative immediate early (IE), early, and late proteins throughout a time course of infection. The IE protein IE1 was efficiently expressed in control and eIF4AIII depleted cells at all times after infection (Fig. 2A). In contrast the expression of the HCMV early protein pUL44 was reduced and the HCMV late protein pp28 was significantly diminished in eIF4AIII depleted cells.

The HCMV pUL44 gene is required for viral DNA accumulation (Pari et al., 1993); therefore the reduced pUL44 levels in eIF4AIII-depleted cells suggested that eIF4AIII was necessary for efficient viral DNA replication. Control or eIF4AIII-depleted cells were infected with HCMV, and viral DNA accumulation was measured by qPCR. As a positive control, infected cells were treated with phosphonoacetic acid (PAA), a chemical inhibitor of the viral polymerase (Huang, 1975). Depletion of eIF4AIII reduced viral DNA accumulation over 40 fold as compared to control cells (Fig. 2B). We conclude that eIF4AIII is required for efficient HCMV DNA accumulation.

HCMV late gene transcription requires viral DNA replication. Therefore the defect in viral DNA accumulation in eIF4AIII-depleted cells suggested that late gene transcription would be decreased. Consistent with the defect in viral DNA replication, depletion of eIF4AIII diminished transcription of the HCMV late gene UL99, which encodes the pp28 protein, by fivefold (Fig. 2C). Thus eIF4AIII is required for efficient transcription of an HCMV late transcript, likely due to the requirement for eIF4AIII for efficient early gene expression and viral DNA replication.

eIF4AIII is required for efficient nuclear export of viral transcripts

As a core component of the EJC, eIF4AIII facilitates the export of spliced transcripts from the nucleus to the cytoplasm (Singh and Lykke-Andersen, 2003). To determine if eIF4AIII facilitates the export of HCMV mRNAs, we measured the nuclear to cytoplasmic ratio of viral mRNAs in eIF4AIII-depleted cells. Control or eIF4AIII-depleted cells were infected

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with HCMV and fractionated into nuclear and cytoplasmic fractions. The purity of the cytoplasmic and nuclear fractions was monitored by Western blot using antibodies specific for tubulin and lamin A/C, respectively (Fig. 3A). The decrease in tubulin loading was not consistently observed. The nuclear to cytoplasmic ratio of representative IE, early, and late viral mRNAs was then measured by qRT-PCR. This approach measures the relative distribution of an mRNA between the two compartments, and is independent of the total abundance of the transcript. eIF4AIII depletion reduced the accumulation of each viral mRNA in the cytoplasm by 50% as compared to control cells. Interestingly eIF4AIII depletion inhibited the cytoplasmic accumulation of both spliced (IE1) and unspliced (UL44 and UL99) viral mRNAs (Fig. 3B). We conclude that eIF4AIII is necessary for the efficient accumulation of HCMV transcripts in the cytoplasm during infection, suggesting a role for eIF4AIII in the nuclear export of viral mRNAs.

eIF4AIII is dispensable for the translation of HCMV mRNAs

eIF4AIII also stimulates mRNA translation in the cytoplasm during the pioneer round of translation (Hwang et al., 2010; Le Hir and Seraphin, 2008). To determine if eIF4AIII is required for translation of HCMV mRNAs, we measured the effect of eIF4AIII depletion on the association of cytoplasmic viral mRNAs with polysomes. mRNAs that are actively undergoing translation are bound by multiple ribosomes, or polysomes, which can be separated from single ribosomes and ribosomal subunits by centrifugation through linear sucrose gradients. The extent to which an mRNA is being translated can be inferred from the distribution of the mRNA throughout the sucrose gradient. Importantly, only cytoplasmic mRNAs are analyzed in this assay, allowing us to separate the effect of eIF4AIII on viral mRNA expression and nuclear export from its role in the translation of viral mRNAs.

We first determined if depleting cells of eIF4AIII affected polysome abundance in HCMV infected cells. Cytoplasmic extracts of infected control or eIF4AIII-depleted cells were resolved through linear sucrose density gradients and the distribution of ribosomal RNA (rRNA) throughout the gradient was determined by gel electrophoresis as previously described. Fig. 4A shows that polysomes were present in eIF4AIII-depleted cells, and that qualitatively polysome abundance appeared slightly reduced in the absence of eIF4AIII. These results are consistent with previous studies showing that eIF4AIII depletion does not result in an overall decrease in protein synthesis (Choe et al., 2014).

We next determined the effect of eIF4AIII depletion on the translation of specific HCMV mRNAs by measuring their distribution throughout the sucrose gradient by qRT-PCR. While we observed a slight increase in the amount of the IE1 mRNA in the fractions containing monosomes (Fig. 4B, fractions 5–7), the difference between control and eIF4AIII-depleted cells was not statistically significant. Similarly the abundance of IE1 mRNA in the fractions containing heavier polysomes (fractions 11–14) was unchanged. The distribution of cytosolic UL99 mRNA in the sucrose gradient was also similar in control and eIF4AIII-depleted cells (Fig. 4C). These results suggest that eIF4AIII is not required for the efficient association of viral mRNAs with ribosomes in the cytoplasm.

To ensure that the migration of the viral mRNAs in the gradient reflected their association with polysomes rather than a potential interaction with HCMV virions, we determined if

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EDTA treatment altered the migration of the viral mRNA in the gradient. EDTA chelates magnesium cations that are required to maintain ribosome integrity (Arnstein et al., 1965). EDTA treatment shifted both ribosomal RNAs and viral mRNAs to the lighter fractions (Fig. S2), demonstrating that the migration pattern of viral mRNAs in the sucrose gradient reflected their association with ribosomes.

Pateamine A, a natural product inhibitor of eIF4A helicases, has potent antiviral properties

We previously found that treating HCMV-infected cells with hippuristanol, a small molecule inhibitor of the eIF4AI/II helicases inhibited HCMV replication (Lenarcic et al., 2014). Our current data suggested that eIF4AIII is also important for virus replication. We therefore reasoned that an inhibitor of all three eIF4A family members might significantly impair virus growth. One such inhibitor is the natural product pateamine A (Bordeleau et al., 2006; Hood et al., 2001; Low et al., 2007b), which specifically binds and inhibits all three eIF4A helicases (Bordeleau et al., 2005; Korneeva, 2007; Kuznetsov et al., 2009; Low et al., 2007a). Importantly, pateamine A is minimally toxic to quiescent cells, bioavailable and well-tolerated in vivo for extended periods (Di Marco et al., 2012; Kuznetsov et al., 2009). A complete synthesis reaction has been developed for pateamine A, resulting in a stabilized derivative, des-methyl, des-amino pateamine A (Kuznetsov et al., 2009) (DMDA-PatA; further referred to as PatA). We therefore determined if PatA has antiviral properties in addition to its proven efficacy as a chemotherapeutic agent (Lenarcic et al., 2014).

We first determined if PatA was toxic to primary fibroblasts. Concentrations of PatA up to and including 100 nM did not induce cytotoxicity over 120 h (Fig. 5A). We next determined the effect of PatA treatment on HCMV replication in an endpoint assay. Different concentrations of PatA were added at the time of infection, and the levels of cell free virus were measured 120 h later. While the IC50 of PatA was not precisely determined, concentrations of PatA as low as 10 nM limited HCMV replication (Fig. 5B), and no cell free virus was observed at a concentrations of 100 nM. We also measured the effect of 100 nM PatA treatment throughout a single virus replication cycle. When PatA was added at the time of infection, the levels of cell free virus remained below the limit of detection at all times after infection (Fig. 5C). We conclude that PatA inhibits HCMV replication in a dose-dependent manner at concentrations that are not toxic to uninfected cells.

To determine where in the viral life cycle PatA inhibited HCMV replication we examined the effects of PatA on viral protein expression over a single viral replication cycle. 100 nM PatA added at the time of infection reduced the expression of the IE1 protein at 24 h after infection, and IE1 levels remained reduced throughout the time course of infection (Fig. 6A). PatA also delayed and reduced the accumulation of an HCMV early (pUL44) and late (pp28) protein. Consistent with the defect in early viral protein expression PatA inhibited viral DNA accumulation (Fig. 6B).

We next measured the effect of PatA on viral protein accumulation when added to cells after infection had already been established. Cells were first infected with HCMV, and PatA was added at the different times after infection for a twenty-four hour period. Untreated controls were harvested at the beginning and end of each treatment window. The addition of PatA at any time after infection prevented further increase in the abundance of viral proteins (Fig.

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7A). For example, the HCMV late protein pp28 was first detected at low levels at 48 h after infection in untreated cells. While pp28 levels increased between 48 and 72 h in untreated cells, the addition of PatA at 48 h prevented further accumulation of pp28 protein. Similar results were observed for the HCMV early protein pUL44. PatA did not limit IE1 expression later in infection likely due to the long half-life of the IE1 protein (Teng et al., 2012) rather than the continued synthesis of IE1 in the presence of PatA.

We also found that adding PatA at any time after infection prevented further accumulation of cell free virus (Fig. 7B). Virus was first detected in the supernatant of untreated samples at 72 h after infection. In untreated cells, the amount of cell free virus increased 50 fold between 72 and 96 h. When PatA was added at 72 h, there was no increase in virus production over the twenty four hour period. Similar results were found when PatA was added from 96 to 120 h after infection. We conclude that PatA is capable of inhibiting HCMV replication during any stage of the lytic cycle.

Discussion

Our results show that the eIF4AIII RNA helicase is required for progression from the immediate early to early stage of the HCMV lytic cycle and for the efficient production of progeny virus. The defect in virus replication in eIF4AIII-depleted cells correlates with our finding that eIF4AIII is required for the efficient export of viral mRNAs from the nucleus. However we find that eIF4AIII is dispensable for the association of viral mRNAs with ribosomes in the cytoplasm. Building on our previous results showing that inhibitors of eIF4AI/II can limit HCMV replication, we found that a chemical inhibitor of all three eIF4A family helicases, pateamine A, potently inhibited HCMV replication at doses that were not toxic to uninfected cells. Importantly, PatA inhibited viral protein synthesis and the further production of progeny virus at any stage of infection. Our results therefore identify eIF4AIII as a novel target for the development of new host-directed antiviral therapeutics.

As a core component of the EJC, eIF4AIII facilitates the export of spliced host mRNAs from the nucleus. Our data shows that eIF4AIII is also required for the efficient nuclear export of HCMV mRNAs, as eIF4AIII depletion reduced the levels of viral mRNAs in the cytoplasm (Fig. 3). This may reflect a requirement for the EJC in the nuclear export of viral mRNAs. Interestingly, the EJC accessory protein UAP56 binds to the HCMV UL69 protein (pUL69) and facilitates the nuclear export of unspliced viral mRNAs (Gatfield et al., 2001; Zielke et al., 2012). Perhaps pUL69 acts in concert with the EJC to traffic HCMV mRNAs to the cytoplasm. Such an association could explain our finding that eIF4AIII was necessary for efficient nuclear export of both spliced and unspliced viral transcripts. Alternatively, HCMV could utilize eIF4AIII an EJC-independent mRNA export pathway. However our results cannot exclude the possibility that eIF4AIII is necessary for the expression a short-lived viral or cellular protein needed to traffic HCMV mRNAs. Future studies examining the role of additional EJC proteins and their potential interactions with viral factors will be needed to discriminate between these possible roles.

We found eIF4AIII depletion resulted in a greater than 100 fold defect in virus replication. How might eIF4AIII facilitate virus replication? eIF4AIII depletion decreased the

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cytoplasmic accumulation of the IE1 mRNA (Fig. 3B) but did not significantly impact IE1 protein levels. This likely reflects the high level of transcription of the IE1 mRNA and the long half-life of the IE1 protein, which together allow for efficient IE1 protein expression despite the reduction in cytosolic IE1 transcript. eIF4AIII depletion also reduced nuclear export of the UL44 early transcript and decreased UL44 protein (pUL44) levels (Fig. 2A), likely explaining the reduced viral DNA accumulation (Fig. 2B) in eIF4AIII-depleted cells. Consistent with the defect in viral DNA replication, eIF4AIII depletion reduced the abundance of the UL99 late transcript (Fig. 2C) and further limited the accumulation of UL99 transcripts in the cytoplasm. Together our results suggest a model where eIF4AIII is required for transition from the immediate early to early stage of infection. The reduction in early protein synthesis when eIF4AIII is depleted limits viral DNA replication resulting in decreased expression of viral late transcripts, which also require eIF4AIII for their efficient nuclear export. Thus our results suggest a model where the defect in the nuclear export of viral mRNAs in eIF4AIII-depleted cells results in a cascading effect, which culminates in greatly reduced late protein expression and infectious virus production. However it is important to note that eIF4AIII could also be required for the expression of unknown host or viral factors needed for HCMV replication.

We also found that while eIF4AIII depletion limited HCMV mRNA nuclear export, the remaining viral RNAs that reached the cytoplasm in eIF4AIII-depleted cells efficiently associated with polysomes. This suggests that that eIF4AIII is not required for the efficient translation of viral mRNAs (Fig. 4). Together with the CBC, eIF4AIII stimulates mRNA translation during the pioneer round of translation (Lejeune et al., 2002). While the role of the pioneer round in HCMV protein synthesis has not been tested, our data suggest that if HCMV mRNAs utilize a pioneer round of translation, eIF4AIII is not required for this process.

Based on our previous work showing eIFAI/II inhibition or depletion limited HCMV replication and our current data demonstrating a requirement for eIF4AIII, we determined if pateamine A (PatA), a biologically available inhibitor of all three eIF4A helicases, might have antiviral properties. We found that PatA decreased HCMV replication below the limit of detection when present from the start of infection at concentrations that were not toxic to uninfected cells. We previously showed that the eIF4AI/II inhibitor hippuristanol potently inhibited HCMV replication when added at the time of infection, but virus replication became resistant to hippuristanol during the late stage of infection. In contrast PatA inhibited HCMV replication during any stage of the virus lytic cycle. Together these results suggest that the inhibition of HCMV replication by PatA is likely due to the combined inhibition all three members of the eIF4A helicase family. PatA likely suppresses virus replication by inhibiting multiple eIF4AI/II- and eIF4AIII-dependent steps in the virus replicative cycle, resulting in a profound defect in HCMV replication and viral protein expression.

Our results raise the possibility that PatA or similar drugs might be useful as antiviral drugs. PatA is an effective in vivo chemotherapeutic (Kuznetsov et al., 2009), prevents cachexia (Di Marco et al., 2012) in small animal models, and is well-tolerated in mice for extended periods of time (Kuznetsov et al., 2009). This suggests that PatA might be useful in vivo to

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limit HCMV disease. In addition we find that PatA has antiviral activity against diverse RNA and DNA viruses, suggesting a potential use for PatA as a broad spectrum antiviral (Ziehr and Moorman, unpublished observation). Our results therefore expand the potential uses for PatA or similar eIF4A family inhibitors to the treatment of viral disease.

Materials and methods

Cells, viruses, and reagents

Primary human foreskin fibroblasts (HFFs) were grown in DMEM containing 10% newborn calf serum. Cells were used between passage 5 and 15 in all experiments. Replication defective lentiviruses expressing eIF4AIII-specific shRNAs (TRCN 0000061855 or 0000061856) were generated as described previously (Lenarcic et al., 2014). HFFs were transduced with lentivirus overnight in the presence of polybrene (4 μg/ml). The next day the media was replaced with serum free DMEM, and the cells were used for experiments at 72 h after transduction. Efficient eIF4AIII depletion was routinely assessed by quantitative real-time PCR (qRT-PCR) or Western blot.

A variant of the HCMV AD169 strain containing a green fluorescent protein expression cassette driven by the SV40 promoter (ADGFP; Wang et al., 2004) was used as the wild type virus strain in all studies. Unless otherwise noted, all infections were performed at a multiplicity of infection (MOI) of three in a minimal volume of serum free media. Infected cells were harvested by scraping and stored at −80 °C until analyzed. Quantification of cell-free virus by the TCID50 method was performed as described previously (Moorman and Shenk, 2010).

DMDA-pateamine A (PatA) (Kuznetsov et al., 2009), a synthetic analogue of pateamine A, was generously provided by Dr. Daniel Romo (Texas A&M University).

Preparation of nuclear and cytosolic fractions

Infected cells were harvested by scraping, pelleting, and resuspending in fractionation buffer (20 mM Tris–HCl, pH 7.4, 140 mM KCl, 5 mM $MgCl_2$, 1% Triton-X 100, 10 mM DTT). The lysate was passed five times through a 27 gauge needle, and then centrifuged at 1150 × *g* for five minutes. The resulting supernatant contained the cytosolic fraction, while the pellet contained the nuclear fraction. A portion of each fraction was analyzed by Western blot using antibodies specific for tubulin or lamin A/C to monitor the purity of the cytoplasmic and nuclear fractions, respectively.

Analysis of polysomes and polysome-associated mRNAs

Polysome isolation and analysis were performed as described previously (Lenarcic et al., 2014). Briefly, cells were treated with cycloheximide (CHX; 100 μg/ml) in media for ten minutes, washed twice in PBS containing CHX, and collected by scraping. The cells were pelleted by centrifugation at 5000 × *g* for ten minutes, and then resuspended in 1 ml of polysome lysis buffer (20 mM Tris–HCl, pH 7.4, 140 mM KCl, 5 mM $MgCl_2$, 1% Triton-X 100, 10 mM DTT) containing CHX. Cells were incubated on ice for ten minutes and then disrupted by five passages through a 27 gauge needle. Nuclei were removed by

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centrifugation for five minutes at 1150 × *g*. The supernatant was cleared of mitochondria by centrifugation at 21,000 × *g* for ten minutes, and then layered onto a 10–50% linear sucrose gradient (made in polysome lysis buffer) containing CHX. The gradients were centrifuged for two hours at 4 °C in a SW41 swinging bucket rotor at 35,000 rpm with no brake. After centrifugation the gradients were manually fractionated from the top of the gradient into 750 μl fractions. RNA was extracted from a portion of each gradient fraction and ribosomal RNA (rRNA) was visualized on 2% agarose gels.

For quantification of viral mRNAs in each fraction of the sucrose gradient, total RNA was extracted with Trizol from an equal volume of each gradient fraction. The RNA was then treated with DNase (Ambion), and reverse transcribed to cDNA as described below. The abundance of the specified mRNA in each gradient fraction was quantified by qRT-PCR by comparison to a standard curve as described previously (Terhune et al., 2010). The amount of the RNA in all fractions was then summed to determine the total copies present in the gradient. The amount of the RNA in each fraction is shown as the percentage of the total copies present in the entire gradient.

Quantification of nucleic acids and proteins

DNase-treated RNA was isolated as above, and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). qRT-PCR was performed on a Roche LC480 using SYBR Green master mix and primers specific for the indicated mRNA as described previously (Ziehr et al., 2015).

To quantify viral DNA accumulation, DNA was extracted from infected cells by phenol:chloroform extraction as described previously (Moorman and Shenk, 2010). Briefly, 500 ng of DNA were analyzed by quantitative real-time PCR (qPCR) using primers specific for the HCMV major immediate early promoter. The abundance of viral DNA was determined by comparison to a standard curve consisting of 10^8–10^1 copies of genomic HCMV DNA. In all experiments the R^2 value of the standard curve exceeded 0.98. The results were normalized to the abundance of GAPDH in the sample to correct for variations in loading.

To measure the nuclear export of viral mRNAs, RNA was extracted from the nuclear and cytoplasmic fractions of infected cells as described above. The abundance of each transcript in the nuclear and cytoplasmic fractions was determined by qRT-PCR using a standard curve, and normalized to the abundance of the transcript in total RNA isolated from an aliquot of the sample prior to fractionation. The nuclear to cytoplasmic ratio for each mRNA in control cells expressing scrambled shRNA was set to one.

To measure protein abundance cells were collected by scraping and frozen as dry pellets at −80 °C until use. Cells were lysed in RIPA buffer (50 mM Tris–HCl: 50 mM, pH 7.4, 150 mM NaCl, 1% NP-40: 1%, 0.25% deoxycholate, 1 mM EDTA) and the protein concentration determined by the Bradford assay. Equivalent amounts of protein were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham). For monoclonal antibodies membranes were blocked for one hour at room temperature in 5% milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) before incubation with

primary antibody in TBST-T with 1% BSA for one hour at room temperature or overnight at 4 °C. For polyclonal antibodies, membranes were blocked in 5% milk followed by incubation with primary antibody overnight at 4 °C in 5% BSA in TBS-T. Antibodies to the following proteins were used in this study: IE1 (Zhu et al., 1995; 1:10,000), UL44 (Virusys; 1:1000), pp28 (Silva et al., 2003; 1: 5000), eIF4AIII (Bethyl A302-980A), tubulin (Sigma 1:50,000), lamin A/C (Abcam 1:1000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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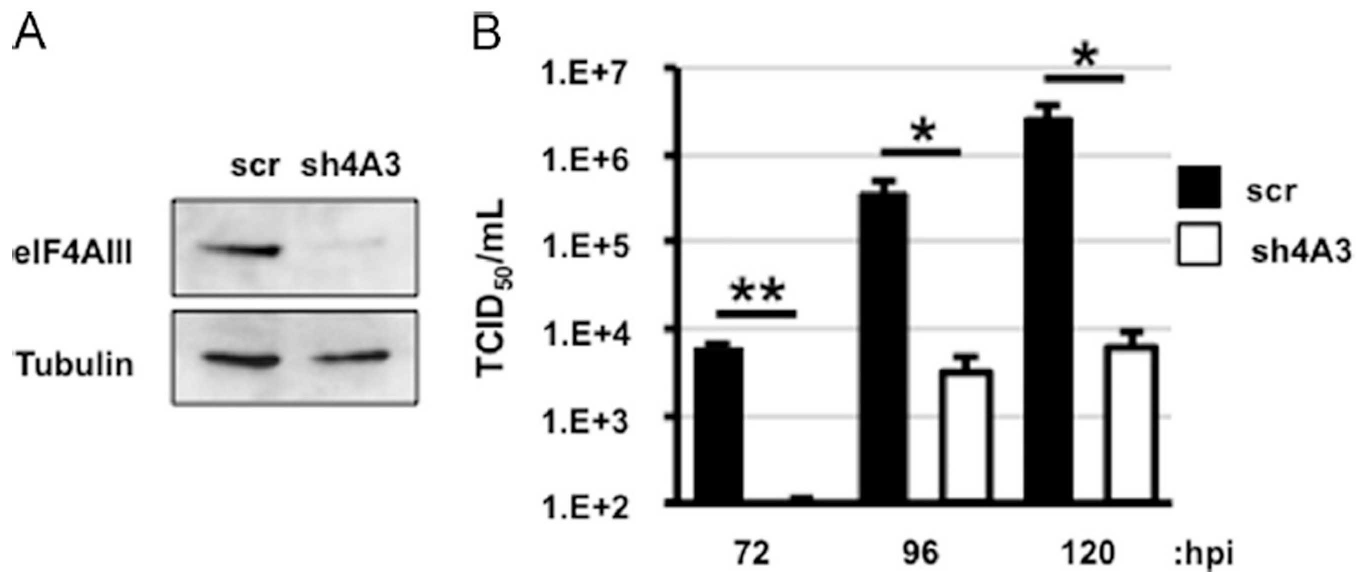

Fig. 1.

eIF4AIII is required for efficient HCMV replication. (A) Primary human fibroblasts were transduced with lentiviruses expressing a scrambled (scr) or eIF4AIII-specific (sh4A3) shRNA and then infected with HCMV (MOI of 3). (A) Representative Western blot demonstrating reduced eIF4AIII protein expression at 72 h postinfection. (B) Cell-free supernatants were harvested at the indicated time and quantified by the TCID50 method (closed bars = scrambled shRNA, open bars = eIF4AIII-specific shRNA; $n = 3$; $*p < 0.05$; $**p < 0.01$).

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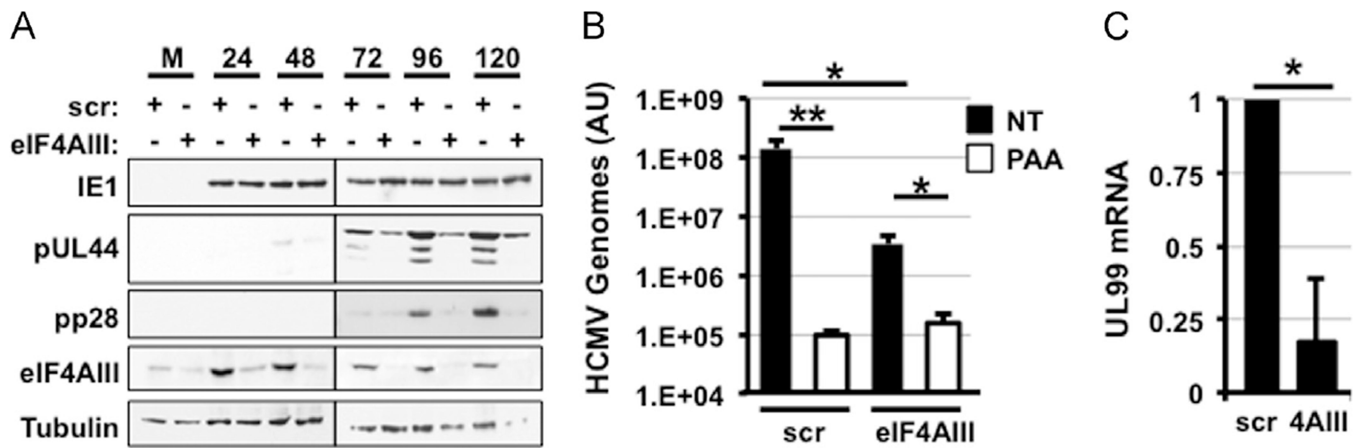

Fig. 2.

Characterization of the HCMV lytic cycle in eIF4AIII-depleted cells. (A) Cells were transduced and infected as in Fig. 1. Protein expression was measured by Western blot (n = 3). (B) Cells were transduced as in Fig. 1 and then infected with HCMV at a multiplicity of 0.05. Viral DNA was quantified at 96 h after infection by quantitative real-time PCR (n = 3; filled bars = untreated, open bars = PAA treated). (C) Cells were transduced and infected as in Fig. 1 and the relative abundance of the UL99 transcript in total RNA was measured at 72 h after infection by quantitative reverse transcriptase real-time PCR (qRT-PCR). The abundance of the UL99 transcript in the scrambled control is set to one (n = 3). For all panels, *$p < 0.05$; **$p < 0.01$.

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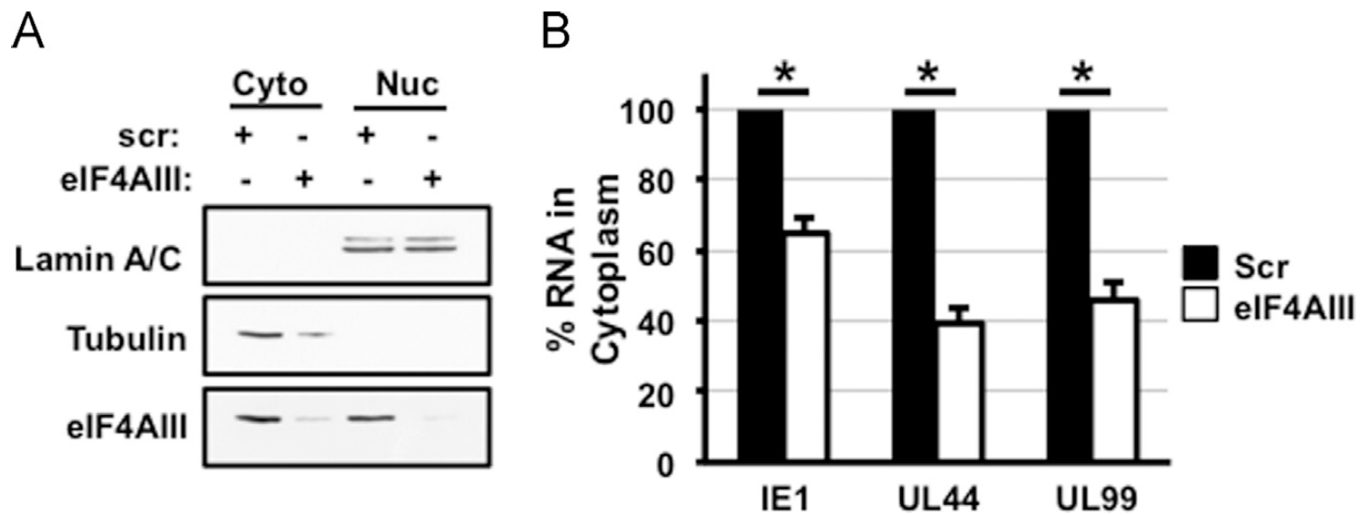

Fig. 3.

eIF4AIII is necessary for efficient accumulation of HCMV mRNAs in the cytoplasm of infected cells. (A) Cells were transduced and infected as in Fig. 1. Seventy two hours after infection the cells were fractionated into nuclear and cytosolic fractions, and the purity of the fractions determined by Western blot using antibodies specific for the cytosolic protein tubulin, and the nuclear protein lamin A/C. (B) As in A, except the cytoplasmic to nuclear ratio of each RNA was determined by qRT-PCR as described in "Materials and methods". The ratio in control cells was set to one ($n = 3$; $*p < 0.05$).

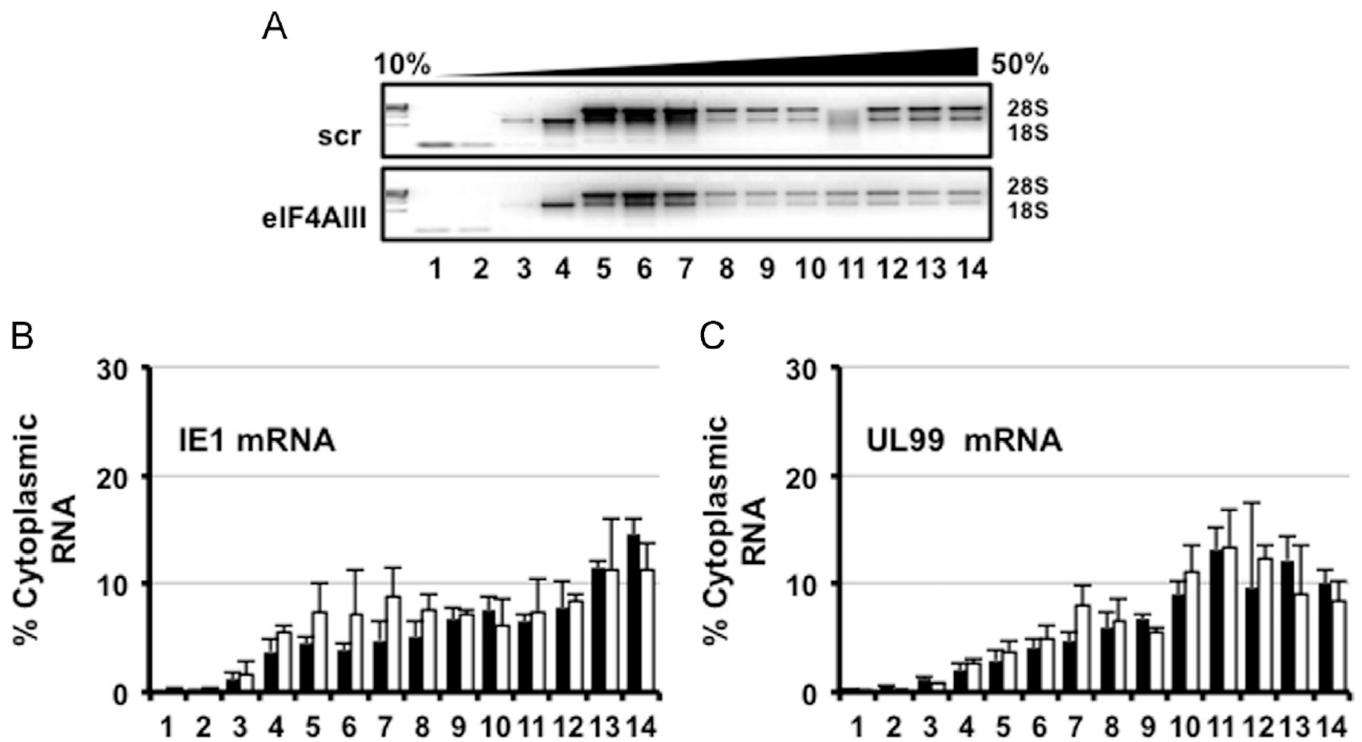

Fig. 4.

eIF4AIII is not required for efficient association of HCMV mRNAs with polysomes. (A) Cells were transduced and infected as in Fig. 1. Seventy two hours after infection cytosolic extracts were resolved through 10–50% linear sucrose gradients. The presence of ribosomal RNAs (28S, 18S) in each fraction was monitored by agarose gel electrophoresis. Monosomes were present in fractions 5–7, while heavy polysomes were present in fractions 10–14. (B and C) As in A, except the abundance of the indicated viral mRNAs in each gradient fraction was determined by qRT-PCR ($n = 3$; closed bars = scrambled; open bars = eIF4AIII depleted cells).

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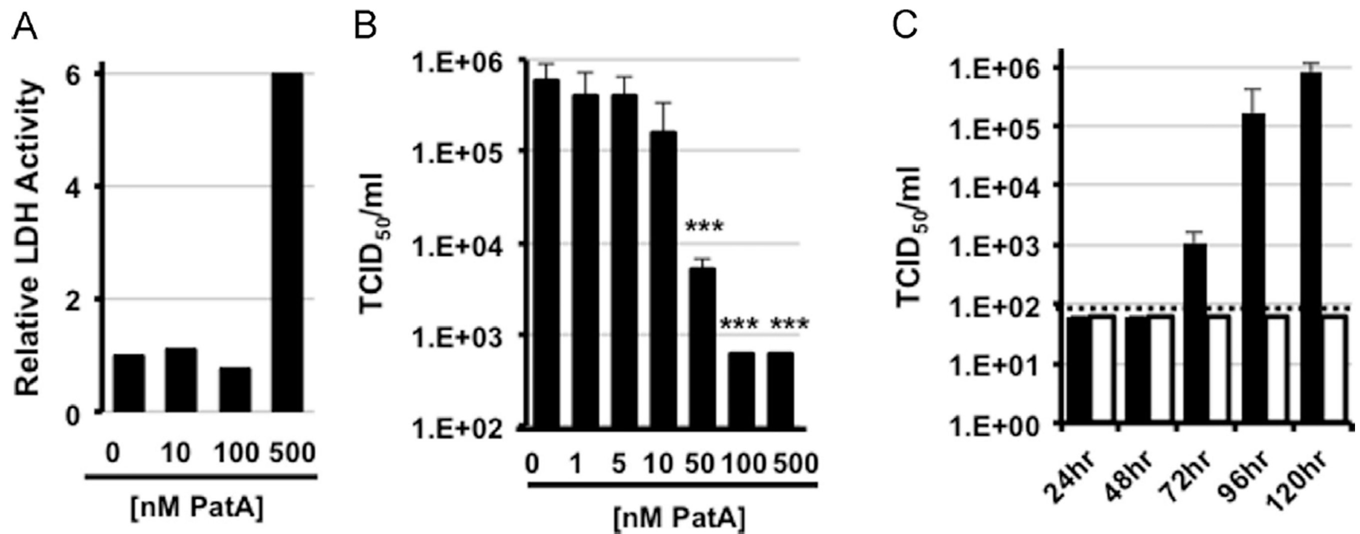

Fig. 5.

Pateamine A inhibits HCMV replication in a dose dependent manner. (A) Uninfected cells were treated with the indicated concentrations of pateamine A (PatA), and toxicity was measured by the lactate dehydrogenase (LDH) assay 120 h later. The results are representative of at least three experiments. (B) Cells were infected as in Fig. 1, and treated with the indicated concentrations of PatA at the time of infection. The amount of cell free virus in the supernatants was quantified at 120 h after infection by the TCID50 method ($n = 3$; ***$p < 0.001$). (C) Cells were infected as in Fig. 1, and treated with PatA (100 nM) at the time of infection. The amount of cell free virus in the supernatants was determined by the TCID50 method (closed bars = vehicle, open bars = PatA; $n = 3$; dashed line indicates limit of detection).

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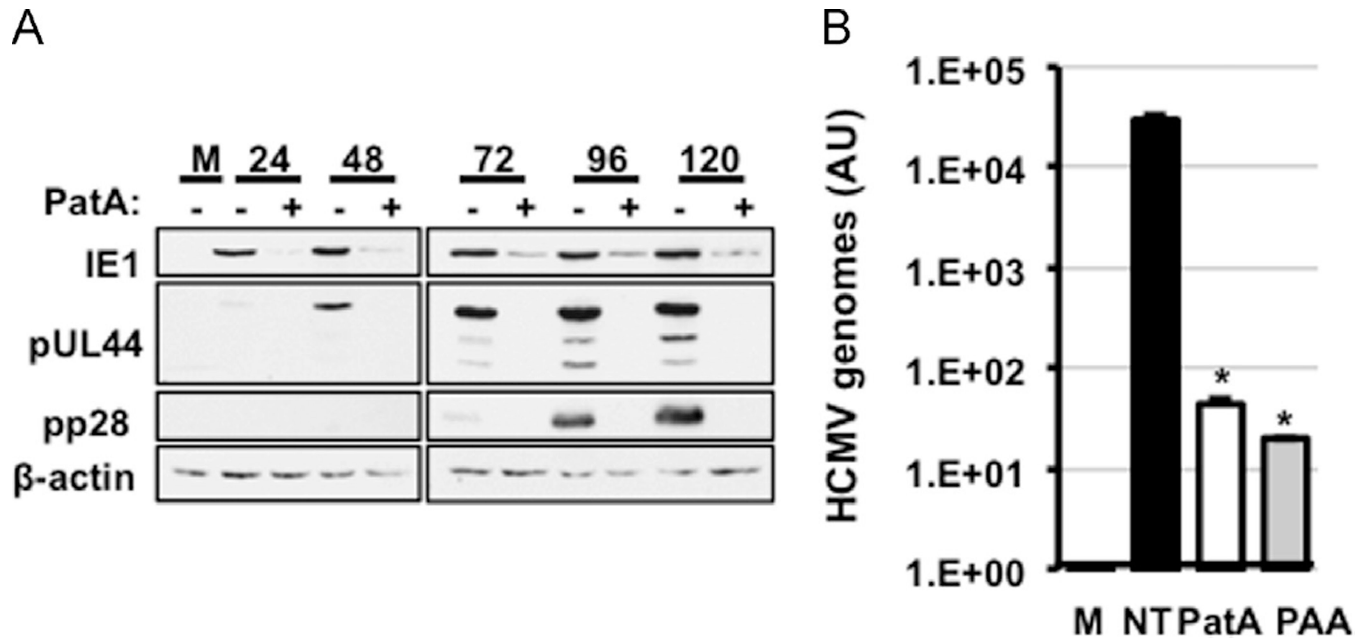

Fig. 6.

Pateamine A inhibits HCMV replication. (A) Cells were infected at a multiplicity of three in the presence of 100 nM PatA. Viral protein expression was measured at the indicated times by Western blot. (B) Cells were infected with HCMV at a multiplicity of 0.05 and treated as in A. Viral DNA was quantified at 96 h after infection by quantitative real-time PCR ($n = 3$).

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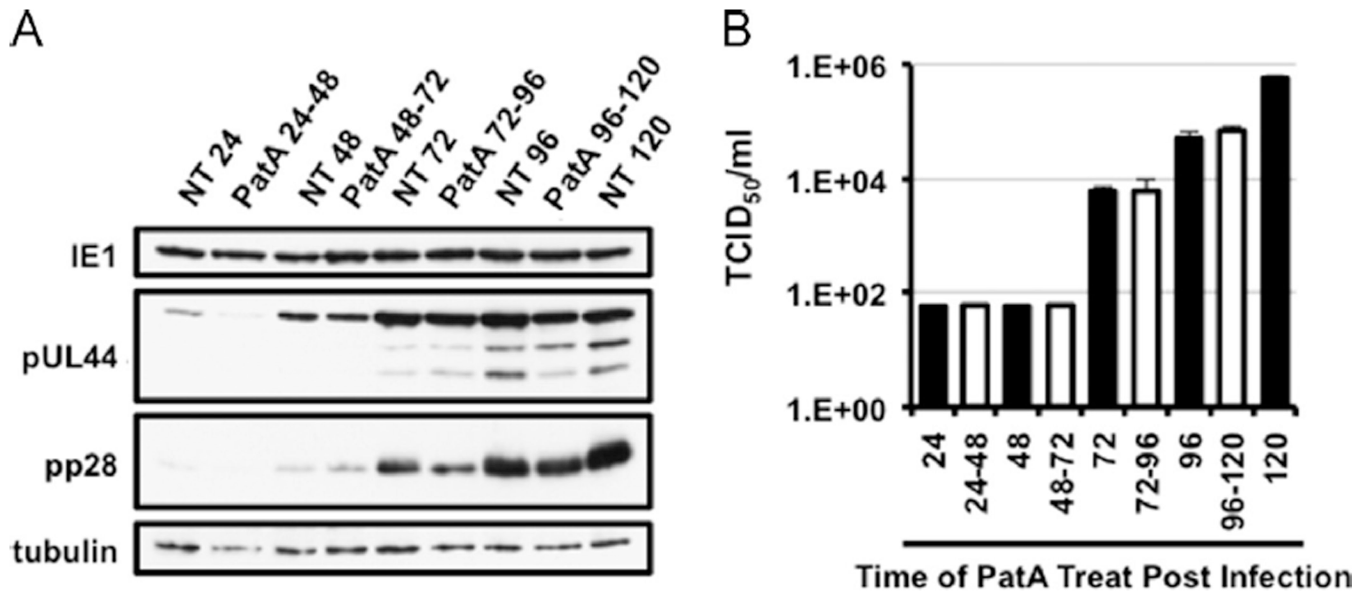

Fig. 7.

Pateamine A inhibits HCMV replication throughout the virus lytic cycle. Cells were infected with HCMV (MOI of 3) and then treated with PatA (100 nM) for the indicated 24 h period. Vehicle treated cells were harvested at the beginning and end of drug treatment as controls. (A) The accumulation of the indicated viral proteins during PatA treatment was measured by Western blot. (B) Cells were infected and treated as in A, and free virus in the supernatants was quantified by the TCID50 method (closed bars = vehicle control, open bars = PatA; n = 3).