Rho-Associated Coiled-Coil Kinase 1 Translocates to the Nucleus and Inhibits Human Cytomegalovirus Propagation

ROCK1 translocates to the nucleus and inhibits human cytomegalovirus propagation

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Abstract

Rho-associated coiled-coil kinase (ROCK) protein is a central kinase that regulates numerous cellular functions, including cellular polarity, motility, proliferation and apoptosis. Here, we demonstrate that ROCK has antiviral properties and inhibition of its activity results in enhanced propagation of human cytomegalovirus (HCMV). We show that during HCMV infection ROCK1 translocates to the nucleus were it localizes adjacent to the viral replication compartments and co-localizes with the Virus-Induced Chaperone-Enriched (VICE) domain marker Hsc70. We further reveal that inhibition of myosin, one of the central targets of ROCK, also increases HCMV propagation, implying that the anti-viral activity of ROCK is mediated by nuclear activation of the actomyosin network. Finally, we demonstrate that inhibition of ROCK results in more capsid accumulation in the cytoplasm compared to the nucleus, indicating ROCK activity might inhibit the efficient egress of HCMV out of the nucleus. Altogether our findings illustrate ROCK activity restricts HCMV propagation and suggest this inhibitory effect is mediated by suppression of capsid egress out of the nucleus.

Importance

ROCK is a central kinase in cells that regulates numerous cellular functions, including cellular polarity, motility, proliferation and apoptosis. Here we reveal a novel anti-viral activity of ROCK1 during infection with HCMV, a prevalent pathogen infecting most of the population worldwide. We reveal ROCK activity is exerted by translocation to the nucleus where it localizes to discrete domains, which are reminiscent of the Virus-Induced Chaperone-Enriched (VICE) domains described in HSV-1. Our findings suggest that ROCK's anti-viral activity takes place via nuclear activation of the actomyosin network and leads to inhibition of capsid egress out of the nucleus.

Introduction

As obligate intracellular parasites, viruses require the host cell machineries and resources to replicate and propagate. In response, mammalian cells have evolved elaborate defense mechanisms to detect and inhibit viral replication. The innate and acquired immune systems are effective at reducing the burden of viral disease but additional cellular activities provide protection from viruses. These functions are mediated by cellular proteins that are consecutively expressed and called restriction factors. In some cases, restriction of viral replication may result from a cell-regulatory function rather than direct interference with the viral replication cycle (1).

We previously integrated translation efficiency measurements with measurements of protein abundance along HCMV infection to identify 65 cellular proteins that presented profiles which suggested they are targeted for degradation during HCMV infection (2). Since targeted degradation may indicate biological importance, we hypothesized that some of these proteins may act as novel HCMV restriction factors. One of the proteins we identified was the Rho-associated coiled-coil kinase (ROCK)1 protein. ROCK1 and its homologue ROCK2 are serine/threonine kinases that were initially identified as activated Rho (Rho-GTP) interacting proteins (3) and are known today to be the major downstream effectors of the small GTPase RhoA. ROCKs can also be activated, independently of Rho, by several lipids and by oligomerization, possibly through amino-terminal transphosphorylation (3).

Upon activation, ROCKs function as versatile kinases, regulating a plethora of cellular processes including cellular polarity, motility, proliferation and apoptosis (3–5). One of the best characterized roles of ROCKs is regulation of actin-filament assembly and contractility. This is achieved by phosphorylation of different substrates, including LIM kinase, myosin light chain (MLC) and MLC phosphatase (5) . Phosphorylation of LIM kinase leads to stabilization of actin filaments and phosphorylation of the myosin light chain (MLC) and inactivation of MLC phosphatase enhance the activity of the motor protein myosin. As a consequence, ROCK activity enhances actin-myosin contraction (4, 6).

In this study, we examined ROCK's function during HCMV infection. By using specific inhibitors and genetic knock down we reveal that ROCK activity inhibits HCMV propagation. We further demonstrate that during HCMV infection ROCK1 is recruited to the

nucleus to specific Hsc70 containing-nuclear domains. These domains are reminiscent of Virus-Induced Chaperone-Enriched (VICE) domains characterized in Herpes Simplex Virus 1 (HSV1) infected cells, and were proposed to act as protein quality control centers that sequester misfolded or modified proteins (7). Moreover, we show that ROCK anti-viral activity is probably mediated by hyperactivation of actin-myosin contraction, as the myosin inhibitor, Blebbistatin, also increased HCMV titers. Finally, we demonstrate that inhibition of ROCK activity enhances both viral gene expression and capsid budding out of the nucleus, suggesting that ROCK antiviral activity might be related to the regulation of actomyosin network in the nucleus.

Results

ROCK inhibition promotes HCMV propagation

Integration of protein production levels (as measured by ribosome profiling) with protein abundance measurements along HCMV infection suggested that Rho-associated coiled-coil kinase 1 (ROCK1), a key regulator of actomyosin network and cell polarity, might be degraded during HCMV infection ((2) and Figure S1A). We previously demonstrated that the RNA levels of ROCK1 were increased during HCMV infection whereas ROCK1 protein levels as measured by immunoblotting were reduced ((2) and Figure S1B), supporting our initial hypothesis that ROCK1 might be degraded during HCMV infection. Since targeted degradation may indicate biological importance, we wanted to test whether the activity of ROCK1 is important for HCMV propagation. To this end, we infected fibroblasts with the HCMV Merlin strain and at 12 hours before infection or 5 hours post infection (hpi) cells were treated with a potent ROCK inhibitor, Y27632 (8). Importantly, inhibition of ROCK resulted in more than a 10-fold increase in viral titers (Figure 1A), suggesting ROCK activity inhibits HCMV propagation. We previously showed the reduction in ROCK1 protein level occurred only when cells were infected with HCMV Merlin strain but not when cells were infected with the HCMV laboratory-adapted strain, AD169, in which a 15 kb comprising the ULb' region (genes UL133–UL150) is deleted (2). We, therefore, tested the effect of ROCK inhibition on AD169 propagation. In support of a substantial difference between these two HCMV strains and in agreement with previous findings (9), inhibition of ROCK activity had no effect on AD169 titers (Figure 1A). We next used an HCMV Merlin strain that contains a GFP tagged UL32 (UL32-GFP)(10), which allows for fluorescence-based monitoring of infection by progeny virions. Fibroblasts were infected with UL32-GFP and ROCK inhibitor was added at different times post infection. Supernatants were collected at 5 days post infection (dpi) and used to infect fresh wild type fibroblasts and the percentage of GFP positive cells was measured by microscopy and flow cytometry, as proxy for viral titers. Utilizing this approach, we could show that inhibiting ROCK activity even at 48 hpi can increase viral propagation (Figure 1B and Figure S1C). Furthermore, inhibition of ROCK by a different, more selective inhibitor, H1152 (11), also resulted in increase in viral titers excluding the possibility that the effect we observed is related to off targets effects of the drug

we used (Figure S1D and S1E). We further established the effect of ROCK inhibition on viral titers by knocking down (KD) ROCK expression using siRNAs. We confirmed that ROCK KD led to a significant reduction in ROCK1 protein expression (Figure S1F). In accordance with our findings using drugs, ROCK KD resulted in a significant increase in viral titers following infection with Merlin strain but not with AD169 strain (Figure 1C).

To define the infection stage affected by ROCK activity, infected cells were treated with ROCK inhibitor and viral proteins levels were examined at different time points post infection (Figure 1D). We observed an elevation in viral protein expression (UL44 and pp28) compared to the untreated sample only at 72 hpi, indicating ROCK activity inhibits late stages of HCMV propagation. In agreement with a late inhibitory effect, we did not observe major differences in the levels of viral DNA replication when ROCK activity was inhibited (Figure 1E).

ROCK1 is translocated to the nucleus during HCMV infection

Given ROCK's anti-viral activity, we sought to determine if ROCK1 is indeed degraded in HCMV-infected cells. Cells were infected with Merlin strain and were then treated with inhibitors of the proteasome (MG132) or lysosome (folimycin). Surprisingly, inhibition of proteasomal or lysosomal degradation did not affect the levels of ROCK1 as assessed by immunoblotting (Figure 2A and data not shown), indicating that ROCK1 is not actively degraded by the proteasome or lysosome in HCMV infected cells.

We therefore aimed to confirm the reduction in ROCK1 protein levels detected by western blot analysis, in an alternative method and to inspect its localization during HCMV infection. We probed for ROCK1 expression using immunofluorescence in mock cells and in cells infected with Merlin or AD169 strains. Remarkably, infection with Merlin, but not with AD169, resulted in translocation of ROCK1 into well-defined nuclear domains (Figure 2B). Subcellular fractionation and immunoblotting analysis confirmed that in mock- and AD169- infected fibroblasts, ROCK1 was predominantly located in the cytoplasmic fractions whereas in Merlin infected fibroblasts, a significant portion of ROCK1 was detected in the nuclear fraction (Figure 2C). Notably, the reduction in ROCK1 levels we measured by immunoblotting occurred at a similar time along infection as its nuclear localization (Figure

S2). We further tested if this translocation of ROCK1 into the nucleus occurs in additional cell types. Epithelial RPE-1 cells were infected with Merlin strain and although as expected the infection was very inefficient (12, 13), ROCK1 was also observed in well-defined nuclear domains in cells that were infected (identified by pp28 staining, Figure 2D).

ROCK1 localizes to nuclear insoluble domains

Since our previous immunoblot analysis suggested that ROCK1 protein levels are reduced during HCMV infection ((2) and Figure S1B), we hypothesized that the nuclear puncta we observed by microscopy might be partially insoluble and therefore affected our ability to detect ROCK1 protein in infected cells using immunoblotting. To test this hypothesis, we harvested mock-infected cells and cells infected with Merlin or AD169 HCMV strains, and lysed them using mild or harsh lysis conditions. When using mild lysis conditions, the detection of ROCK1 by immunoblotting was reduced in cells infected with Merlin strain but not with AD169 strain, compared to mock-infected cells (Figure 3A). This reduction in ROCK1 detection was not simply due to inefficient extraction of nuclear fractions as we obtained comparable levels of UL57 (a viral protein that resides in the nuclear replication compartment). When harsh lysis conditions were used we did not detect any reduction in ROCK1 expression (Figure 3A), supporting the assumption that the puncta-localized ROCK1 is partially insoluble.

To further examine if ROCK1 localizes to insoluble nuclear domains during HCMV infection we tested if it could be extracted from cells using a detergent buffer wash, as it was previously demonstrated that nuclear inclusions are resistant to this short detergent extraction (7, 14). Indeed, we observed that in HCMV-infected cells ROCK1 was resistant to detergent treatment (compare Figure 3B lower and upper panels), whereas pp28 and UL57 were efficiently extracted from the cytoplasm and the nucleus (Figure 3B and Figure S3). We conclude that in Merlin-infected cells ROCK1 localizes to nuclear domains that are partially insoluble and therefore resemble nuclear inclusions.

ROCK1 co-localizes with VICE domain marker Hsc70

In HCMV-infected cells, viral gene expression, DNA replication and encapsidation occur in large nuclear structures designated as viral replication compartments. To examine ROCK1 localization relative to the replication compartment we stained HCMV-infected cells for

ROCK1 together with metabolic labeling of nascent viral DNA using 5-ethynyl-2'-deoxyuridine (EdU) that was visualized using Click chemistry or with co-staining for UL57, which is found throughout the viral replication compartment. These co-staining demonstrated that ROCK1 localized to defined regions that are adjacent to the viral replication compartment (Figures 4A and 4B).

In Herpes Simplex Virus type 1 (HSV1) infected cells, it was demonstrated that cellular chaperone proteins such as Hsc70 are translocated to the nucleus and organize into Virus-Induced Chaperone-Enriched (VICE) domains (7, 15–18). Similar to our observations about ROCK1, VICE domains in HSV1 infected cells are formed adjacent to nuclear viral replication compartment and were shown to be resistant to detergent extraction (7). In HCMV infected cells components of the ubiquitin–proteasome system (UPS) were demonstrated to assemble into domains at the periphery of replication compartments (19, 20), suggesting similar structures might be generated during HCMV infection.

We therefore examined whether ROCK1 is localized to structures which are similar to VICE domains. To this end we stained mock- and HCMV-infected cells for Hsc70, a marker that was used to label VICE domains in HSV1 infected cells (15, 16). In HCMV infected cells a portion of the Hsc70 protein was translocated to specific puncta in nucleus and this puncta-localized Hsc70 co-localized with ROCK1 (Figure 4C). These results indicate that ROCK1 localizes to domains similar to VICE domains during HCMV infection.

ROCK inhibition enhances virus egress out of the nucleus

We next wanted to elucidate how ROCK inhibition enhances HCMV propagation. It was previously demonstrated that ROCK inhibition reduces cellular apoptosis (21). Therefore, a simple explanation for the enhanced viral production when ROCK is inhibited could stem from improved cell survival. However, using both PI staining and XTT assay, we did not observe any differences in cell viability when HCMV infected cells were treated with ROCK inhibitor (Figure 5A and Figure S4). ROCK activity enhances intracellular contractive forces via the actomyosin network (22). To test if the anti-viral activity of ROCK is related to actomyosin-mediated contractility we tested the effects of the myosin inhibitor, Blebbistatin, on HCMV propagation. Notably, similarly to ROCK inhibition, inhibition of myosin activity

resulted in enhancement of Merlin but not AD169 propagation (Figure 5B), indicating the anti-viral activity of ROCK might be related to the activation of the actomyosin network.

Since the actomyosin network can affect nuclear organization, we next tested if ROCK inhibition disrupts the formation of VICE-like domains by analyzing Hsc70 localization. Inhibition of ROCK did not affect Hsc70 localization to nuclear domains (Figure 5C), although we verified it abolished the phosphorylation of MLC (Figure S5), illustrating ROCK activity is probably not important for the generation Hsc70 containing domains. Furthermore, we could identify nuclear domains containing Hsc70 in cells infected with the HCMV AD169 strain (Figure 5D), in which ROCK1 is not recruited to the nucleus (Figure 2B), supporting the conclusion that ROCK activity is not involved in the formation of VICE-like Hsc70 containing domains. In contrast, we noticed that inhibition of ROCK activity resulted in less recruitment of ROCK1 itself into the nucleus (Figure 5E), suggesting that ROCK1 translocation to the nucleus is dependent in its activity.

The observation that ROCK inhibition reduced its nuclear localization pointed out that ROCK anti-viral activity is likely related to its nuclear localization. Furthermore, our results indicated that ROCK activity inhibits late stages of HCMV replication and is involved in the activation of the actomyosin network. We therefore sought to determine whether ROCK inhibition affects the nuclear egress of HCMV which was recently shown to depend on nuclear actin filaments (23). Subcellular fractionation and immunoblotting analysis demonstrated that UL32, a tegument protein that associates with HCMV capsids (24), was more abundant in the cytoplasmic fraction when ROCK was inhibited (Figure 5F). This result indicates that inhibition of ROCK activity probably leads to more efficient exit of HCMV capsids out of the nucleus. Over all, our results demonstrate that during infection with HCMV using a wild type strain, such as Merlin, ROCK1 kinase restricts HCMV propagation. We propose a mechanism by which ROCK1 translocates to the nucleus where it activates the actomyosin network and this process inhibits late viral gene expression the efficient exit of HCMV capsids out of the nucleus.

Discussion

In this study, we first reveal that ROCK activity restricts HCMV propagation as inhibition of ROCK resulted in a significant 10-fold increase in viral titers. Interestingly we show that during HCMV infection ROCK1 translocates to the nucleus, where it localizes to nuclear domains that are insoluble and contain Hsc70, therefore show resemblance to the VICE domains that were previously characterized in HSV1-infected cells (7). Although ROCK1 localizes to VICE-like domains, our experiments suggest its activity is not required for these domains' formation, and it is likely that they are functionally independent. Nevertheless, it is still possible that ROCK activity plays a role in recruiting substrates to this still enigmatic compartment. Our experiments do reveal, however, that inhibition of ROCK partially blocks its own recruitment to the nucleus. This result, together with the observations that during infection with HCMV AD169 strain ROCK1 is not recruited to the nucleus and does not affect viral titers, suggests that ROCK anti-viral activity is nuclear.

ROCK's activity was previously studied in the context of infections revealing plethora of effects. In the case of Equine Herpesvirus 1 (EHV1) and HSV1 ROCK inhibition inhibited viral entry (25, 26). For EHV1 the importance of ROCK activity was tested for two different entry pathways, direct fusion and endocytic pathway. In both, ROCK was critical for infection, suggesting ROCK is not required for the initial penetration step but to later stages of entry (26). ROCK activity was also demonstrated to have opposing roles in infection-induced cell motility. It was demonstrated that vaccinia virus induces cell motility through inhibiting both ROCK and mDia (27). In contrast, during HCMV infection of vascular smooth muscle cell, a viral encoded chemokine, US28, was shown to induce cell motility. In this case inhibition of ROCK activity blocked US28-induced cellular migration, suggesting ROCK signaling is important for US28-mediated cellular migration (28). Our observations indicate an anti-viral activity of ROCK, which seems to be unrelated to these previously described infection related functions of ROCK. In support of our findings that ROCK activity inhibits HCMV propagation, a recent siRNA screen that examined the effects of membrane organization factors on HCMV propagation revealed that KD ROCK1 significantly increase HCMV titers (29).

ROCKs act as kinases that phosphorylate various substrates, including MLC and LIM kinase (3) and one of ROCK's central roles is to regulate actomyosin contractility. Interestingly, we reveal that treating HCMV infected cells, following viral entry, with the myosin inhibitor, Blebbistatin, induces viral titers to similar extent as ROCK inhibition. Also in the case of myosin inhibition, increased viral titers were observed only when cells are infected with the Merlin strain but not with the AD169 strain. These results suggest that ROCK anti-viral activity is probably related to its regulation of the actomyosin network.

Actomyosin-mediated contractility is a highly conserved mechanism that generates mechanical stress in animal cells and is involved in many cellular processes such as changes in shape, intracellular transport and cell mechanosensing (30). Our results suggest that the anti-viral activity of ROCK is nuclear and occurs at a late time point of infection. Previously it was shown that tracking UL32 can be used to track viral processes in infected cells, including nuclear egress (31). ROCK inhibition leads to a significant elevation of cytoplasmic UL32 at late time points of infection, suggesting that nuclear egress is inhibited by ROCK activity.

The involvement of nuclear actomyosin network in intranuclear movements of herpesvirus capsids was previously studied and remains controversial (32). HSV1 capsids motility in the nucleus were shown to be antagonized by temperature reduction or by inhibitors of ATP, myosin, or actin (33). In line with these observations it has been shown that HSV1 and pseudorabies virus (PRV) infections result in the formation of nuclear actin filaments and HSV1 capsids were shown to associate with nuclear myosin V (34). In contrast more recent analysis reported that HSV1 and PRV infections remodel nuclear architecture so that capsids can diffuse to the nuclear periphery (35). For HCMV it was demonstrated that nuclear actin filaments are induced during infection and that these actin filaments are important for HCMV nuclear egress (23). Furthermore, HCMV capsids were shown to associate with nuclear myosin V which was required for capsid accumulation in the cytoplasm and for efficient production of infectious virus (36). Our results add another level of complexity to these previous findings as we show that inhibition of ROCK and direct inhibition of Myosin II with Blebbistatin increases viral titers. These results suggest that nuclear actomyosin activity can also suppress HCMV propagation. However, we cannot preclude that this is an indirect effect occurring via other cellular processes, such as the

mechano-state of the nucleus. It is clear that herpesvirus infection and specifically the generation of replication compartment poses some major mechanical constraints on the nucleus. Recent evidence shows that the local mechano-environment within cells can regulate transcription (37). There is also evidence for roles of nuclear actin and myosin in transcription, chromatin remodeling, and mRNA export (38, 39). It is therefore possible that ROCK inhibition relieves a potential stress or constrains on transcription that then affects viral propagation and viral egress out of the nucleus. Supporting this notion of an indirect effect is the increase we observed in viral gene expression at 72 hpi when we inhibit ROCK activity. This increase could not be solely explained by more efficient exit of capsids out of the nucleus.

Another interesting aspect of our findings is the differences we reveal between the HCMV laboratory-adapted strain, AD169, in which a 15 kb composing the ULb' region (genes UL133–UL150) is deleted and the Merlin strain that is considered a WT strain with characterized mutation in only two viral proteins (10). It is well established that there are drastic differences in the entry pathways different HCMV strains are using (40). Our results point there might also be ULb'- dependent differences in the way HCMV bud out of infected cells. Future work will have to delineate the contribution of different viral genes and their association with ROCK1 entry to the nucleus and anti-viral activity.

In summary, we demonstrate that ROCK activity inhibits HCMV propagation at late stages of infection. Our results indicate that this activity is related to nuclear activation of the actomyosin network. Our findings and future studies aimed at resolving the role of the nuclear actomyosin- network for HCMV propagation may be important not just for HCMV biology but also for general understanding of the potential functions of actomyosin in the nucleus.

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Figure legends

Figure 1. ROCK activity inhibits HCMV propagation

(A) HFF cells were infected with Merlin or AD169 HCMV strains and the ROCK inhibitor Y27632 or DMSO (as control) were added 12hr before or 5hr post infection. Supernatants were collected 5 dpi and viral titers were measured by TCID50 assay. (**B)** HFF cells were infected with Merlin UL32-GFP strain and were either treated with Y27632 at 5, 24, 48 and 72 hpi, or treated with DMSO. Supernatants were collected at 5 dpi and were used to infect fresh HFF cells. Viral titers were quantified by measuring percentage of GFP positive cells using FACS. (**C**) HFF cells were transfected with an siRNA pool targeting ROCK1 and 2 or a control siRNA pool and infected with Merlin or AD169 HCMV strains at MOI=3. Supernatants were collected 5 dpi and were used to infect fresh HFF cells. Viral titers were quantified by measuring percentage of GFP positive cells using FACS. (**D-E)** HFF cell were infected with Merlin strain and ROCK inhibitor (Y26732) was added at 5, 24 and 48 hpi. **(D)** Proteins were extracted at the indicated times and analyzed by Western blot analysis with IE1/2, UL44 and PP28 serving as immediate early, early and late gene markers respectively. GAPDH was used as a loading control. **(E)** DNA was extracted 72 hpi and quantified by real-time PCR using primers for UL55. DNA levels were normalized to the human gene B2M. Means and error bars (showing standard deviations) represent triplicates. * p-value<0.05, ** p-value<0.01 by two-sided student's t-test.

Figure 2. ROCK1 re-localizes to the nucleus after infection with HCMV Merlin strain

(A) MG132 was added to mock- or Merlin-infected cells at 72 hpi for 5 hours and ROCK1 levels and MHC Class I, which was used as positive control, were analyzed by Western blot. GAPDH was used as a loading control **(B)** Fluorescent microscopy images of mock-infected HFF cells (top) or HFF cells infected with Merlin (middle) or AD169 (bottom) HCMV strains, and stained with DAPI (blue) and ROCK1 antibody (red) at 72 hpi. **(C)** Subcellular localization of ROCK1 protein was examined by cellular fractionation at 72 hpi, separating between the cytosol and nuclear fractions. Equivalent amount of proteins from the total (T) cytosol (C) and nucleus (N) fractions were analyzed by western blot for ROCK1, GAPDH (cytosolic marker) and histone H2B (nuclear marker). Quantification of the ratios of nuclear

and cytosolic ROCK1 from two independent experiments is presented. Error bars show standard deviations. ** p-value<0.01 by two-sided student's t-test. **(E)** RPE cells were infected with HCMV Merlin strain and at 72 hpi stained with DAPI (blue), PP28 (green) and ROCK1 (red).

Figure 3. ROCK1 in HCMV infected cells is resistant to detergent treatment

(A) Total protein from uninfected HFF cells or HFF cells infected with Merlin or AD169 strains, was extracted 72 hpi using mild or harsh lysis buffers. Protein levels were detected by Western blot analysis for ROCK1, GAPDH and UL57. **(B)** Fluorescent microscopy images of Merlin infected HFF cells treated with either PBS or detergent extraction buffer and stained with pp28 (green) and ROCK1 (red) antibodies and DAPI (blue).

Figure 4. Nuclear ROCK1 localize to HCMV-induced VICE-like domains

(A-B) HFF cells were infected with Merlin strain and stained at 72 hpi for ROCK1 (red) and DAPI (blue). Replication compartments were imaged by either metabolically labeling nascent DNA with ethynyl-2'-deoxyuridine (EDU) and attaching a fluorophore by "Click" chemistry (green) **(A)** or by staining for UL57 (green) **(B)**. **(C)** HFF cells were mock-infected or infected with Merlin strain and stained at 72 hpi for ROCK1 (red), HSC70 (green) and DAPI (blue).

Figure 5. ROCK activity blocks viral egress out of the nucleus

(A) Merlin infected HFF cells were harvested at 5 dpi, stained with PI (Propidium iodide) and analyzed by FACS. Means and standard deviations of triplicates are represent. **(B)** HFF cells were infected with Merlin UL32-GFP or AD169-GFP HCMV strains and ROCK inhibitor (Y27632) or myosin inhibitor (blebbistatin) were added 5 hpi. Supernatant were collected at 5 dpi and used to infect fresh HFF cells. Viral titers were quantified by measuring percentage of GFP positive cells using FACS. Means and standard deviations of triplicates are represent. **(C)** HFF cells were infected with Merlin HCMV strain and treated with ROCK inhibitor (Y26732) or DMSO (as control) at 5 hpi and stained 72 hpi with DAPI (blue) and HSC70

antibody (green). Cells were treated with detergent extraction buffer before staining. The quantification of the percentage of cells containing HSC70 puncta in the nucleus from three independent experiments is presented (n=200). Error bars show standard deviations. **(D)** HFF cells infected with AD169 were stained at 72 hpi with DAPI (blue) and HSC70 antibody (green). **(E)** HFF cells were infected with the Merlin strain and at 5 hpi cells were treated with ROCK inhibitor (Y26732) or DMSO (as control). At 72 hpi the cells were stained with DAPI (blue) and ROCK1 antibody (red). The quantification of the percentage of cells containing ROCK1 puncta in the nucleus from three independent experiments is presented (n=400). Error bars show standard deviations. **(F)** Cells infected with Merlin strain harboring a UL32-GFP were fractionated to separate between the cytosol and the nucleus fractions. Proteins were analyzed by western blot for UL32-GFP, GAPDH (cytosolic marker) and UL57 (nuclear marker). Quantification of the ratios of nuclear and cytosolic GFP from three independent experiments is presented. Error bars show standard deviations. * p-value<0.05, ** p-value<0.01 *** p-value<0.001 by two-sided student's t-test.

Figure S1. ROCK1 inhibits HCMV infection

(A) Ribosome profiling measurements of ROCK1 translation compared with protein abundance (2). **(B)** Analysis of ROCK1 RNA (upper panel) and protein (lower panel) levels. RNA was measured by real time RT-PCR and the levels were normalized to the human transcript MFGE8. ROCK1 protein was measured by western blot analysis. GAPDH was used as a loading control. **(C)** HFF cells were infected at MOI=5 with Merlin UL32-GFP strain and were either treated with ROCK inhibitor (Y27632) at 5, 24, 48 and 72 hpi, or treated with DMSO as negative control. Supernatants were collected 5 dpi and were used to infect fresh HFF cells, which were visualized by fluorescence microscopy. **(D-E)** HFF cells were infected at MOI=5 with Merlin UL32-GFP strain and at 5 hpi were either treated with ROCK inhibitors (Y26732 and H1152) or with DMSO as negative control. Supernatants were collected 5 dpi and were used to infect fresh HFF cells, GFP positive cells which were visualized by fluorescence microscopy **(D)** and quantified by FACS **(E)**. Means and error bars of triplicates are presented. * p-value<0.05, ** p-value<0.01 by two-sided student's t-test. **(F)** HFF cells were transfected with an siRNA pool targeting ROCKs or a control siRNA pool

and infected with Merlin or AD169 HCMV strains. Proteins were extracted at the indicated times post infection and the levels of ROCK1 and IE1/2 were analyzed by western blot analysis. GAPDH was used as a loading control.

Figure S2. The reduction in ROCK1 signal by western blot concurs with its localization to nuclear puncta along infection

A graph showing the relative levels of ROCK1 (compared to mock) along infection as determined by western blot analysis and the percentage of nuclei in which ROCK1 was localized to nuclear puncta as quantified by microscopy (n=200).

Figure S3. ROCK1 in HCMV infected cells is resistant to detergent treatment

Mock- or Merlin- infected cells were treated with either PBS or detergent extraction buffer. Cells were then fixed, permeabilized and the localization of ROCK1 and UL57 was detected by immunofluorescence.

Figure S4. Elevated viral titers induced by inhibition of ROCK are not due to increased cell viability

Merlin infected HFF cells were treated with the ROCK inhibitor Y26732 or with DMSO as control, and cell viability was measured by XTT assay. A representative analysis of two independent experiments is shown. Means and error bars (showing standard deviations) represent five replicates.

Figure S5. Treatment with the ROCK inhibitor Y26732 abolishes MLC phosphorylation

HFF cells were treated with the ROCK inhibitor Y26732 for 6hrs and MLC phosphorylation was assayed by western blot using an antibody for phospho-MLC. GAPDH was used as a loading control.

Materials and methods

Cells, viruses and treatments

Human fibroblasts (CRL-1634), RPE1(CRL-4000) and the HCMV Merlin strain (VR-1590) were obtained from American Type Culture Collection (ATCC). The Merlin UL32-GFP was kindly provided by R. Stanton (41). The AD169 virus was previously described (42–44). The AD169-GFP was kindly provided by M. Messerle (45). Cells were infected at a multiplicity of infection MOI=5, unless stated otherwise, by incubation with the virus for 1hr followed by media replacement.

To achieve ROCK inhibition cells were treated with 10uM Y27632 (sigma) or 2uM H1152 (Santa Cruz) at the indicated times. Myosin was inhibited by treating cells with 2uM of blebbistatin. For proteasome inhibition cells were added with 10um of MG132 for 8h and proteins were extracted and analyzed by western blot.

To test cells viability trypsinized cells were centrifuged at 300g for 5 min and resuspended in 200ul PBS. 0.5µg/ml of *Propidium iodide* (PI) was added, incubated for 1 min and analyzed by FACS. Mock infected cells either untreated or after heat shock treatment of 10min at 65^{o}C served as negative and positive controls, respectively.

$TCID_{50}$ assay

10^4 HFF cells were plated in 96-well plates and cells were infected with 10-fold serial dilutions of supernatant from infected cells, untreated or treated with inhibitor, collected 5 dpi. At 12dpi the dilutions showing cytopathic effect were evaluated by light microscopy. The $TCID_{50}$/ml was calculated using the Spearman-Kaerber method (46).

Knockdown by siRNA

Cells were transfected with siRNA validated for ROCKs (ON-TARGET plus siRNA, Dharmacon) or negative control (IDT) in the presence of Lipofectamine RNAiMAX reagent

(Life Technologies), according to manufacturer's protocol. Infection was performed 24 hours after transfection.

Viral titer measurements using flow cytometry

HFF cells were infected with Merlin UL32-GFP strain or AD169-GFP. 5dpi the supernatant was transferred to fresh HFF cells and 48hpi cells were harvested and percentage of GFP positive cells was measured by flow cytometry and normalized to the relevant control.

Western blot analysis

Cells were lysed using harsh buffer (150mM Sodium Chloride,1% Triton X100, 0.5% Sodium deoxycholate, 50mM Tris pH8.0,0.1% SDS) or mild buffer (150mM Sodium Chloride, 0.2% Triton X100, 50mM Tris pH8.0, 0.1% SDS). Lysates were rotated at 4°C for 10 min and then centrifuged at 20,000× g for 15 min at 4°C. Samples were then separated by 4–12% polyacrylamide Bis-tris gel electrophoesis (Invitrogen), blotted onto nitrocellulose membranes and immunoblotted with primary antibodies; αROCK1 (ab134181 abcam); αGAPDH (2118S, Cell signaling); αUL44 (ICP36) (CA006 Virusys); αpp28 (CA004, Eastcoast); αHistone H2B (ab1790, Abcam); αphospho-MLC2 (C-3674, Cell Signaling Technology). Secondary antibodies were Goat anti-rabbit, Goat anti-mouse (IRDye 800CW or IRDye 680RD, Licor), or Goat anti-Rat (Alexa Fluor 680, ab175778, Abcam). Reactive bands were detected by Odyssey CLx infrared imaging system (Licor). Protein concentration was measured by Bradford assay (Sigma cat no. B6916). Protein quantification was performed using Licor software.

Cellular fractionation

$2x10^6$ HFF cells were seeded in a 10cm plate and either mock-infected or infected with HCMV (MOI 5). At 72 hpi, cells were fractionated using an NE-PER™ kit (thermo Fisher cat 78833). Nuclear and cytoplasmic fractions were separated on SDS gel and analyzed by Western blot.

Immunofluorescence

Cells were plated on ibidi slides and fixed in 4% paraformaldehyde for 15 min, washed in PBS (pH 7.4) and permeabilized with 0.2% Triton X-100 in PBS for 10 min, then blocked with 10% goat serum in PBS for 30 minutes. Immunostaining was performed for the detection of: ROCK1 (abcam 156284) HSC70 (Stressgen SPA-815) UL99 (Eastcoast bio CA004) UL57 (viruses corporation p1209). Cells were washed 3 times with PBS and labeled with the appropriate secondary antibody for 1 hr at room temerature; anti-rabbit Rhodamine Red-X-conjugated (Jackson ImmunoResearch 711-295-152), Cy™2 AffiniPure Rabbit Anti-Human IgG (H+L) (Jackson ImmunoResearch 711-255-152), anti-rat Rhodamine Red-X-conjugated (Jackson ImmunoResearch 711-295-152) and FITC (sigma Anti-mouse IgG F0257). In situ detergent extraction to remove non matrix-bound proteins was performed as previously described (Christine M. Livingston et al, PLos Pathogen 2009). Imaging was performed on a AxioObserver Z1 widefield microscope using a 40x, 63x oil objective and Axiocam 506 mono camera.

EdU staining

EdU staining was performed based on (47). Briefly, HFF cells infected with Merlin for 3 days were incubated with 10uM 5-ethynyl-2’-deoxyuri dine (EdU) (Jena Bioscience GmbH) for 30min. Cells were then fixed with 4% formaldehyde for 10 min, Permeabilized with 0.5% Triton® X-100 for 20 min and stained with staining mix (100mM Tris pH 8.5, 1mM CuSO4, 10uM fluorescent azide, 100mM ascorbic acid) for 30 min. EdU-stained cells were immunostained for ROCK1 by using standard protocol.

Real-time PCR

Total DNA was extracted using QIAamp DNA Blood Mini Kit (51104) according to the manufacturer's protocol. Real time PCR was performed using the SYBR Green PCR master-mix (ABI) on a real-time PCR system StepOnePlus (life technologies) with the following primers (forward, reverse):

UL55; TGGGCGAGGACAACGAA, TGAGGCTGGGAAGCTGACAT

B2M; TGCTGTCTCCATGTTTGATGTATCT, TCTCTGCTCCCCACCTCTAAGT

XTT cell viability assay

HFF cells were seeded in 96-well plates at 5×10^3cells/well, infected the next day with HCMV Merlin strain and treated with the ROCK inhibitor Y26732 or with DMSO as control. 4dpi cells were assayed for cell viability using the XTT-cell proliferation kit (Biological Industries, Beit Haemek, Israel) according to the manufacturer's instructions. The absorbance was measured in an ELISA plate reader at a wavelength of 470 nm and normalized to a background control.

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