The Herpes Simplex Virus 1 vhs Protein Enhances Translation of Viral True Late mRNAs and Virus Production in a Cell Type-Dependent Manner[†]

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The herpes simplex virus 1 (HSV-1) virion host shutoff protein (vhs) degrades viral and cellular mRNAs. Here, we demonstrate for the first time that vhs also boosts translation of viral true late mRNAs in a cell type-dependent manner and that this effect determines the viral growth phenotype in the respective cell type. Our study was prompted by the detection of stress granules, indicators of stalled translation initiation, in cells infected with vhs mutants but not in wild-type-virus-infected cells. Accumulation of true late-gene products gC and US11 was strongly reduced in the absence of vhs in HeLa cells and several other restrictive cell lines but not in Vero and other permissive cells and was independent of phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2 α). Polysome analysis showed that gC and US11 transcripts were poorly translated in vhs-null-virus-infected HeLa cells, while translation of a cellular mRNA was not affected. Interestingly, hippuristanol, an eIF4A inhibitor, produced a similar phenotype in HeLa cells infected with wild-type HSV-1, while Vero cells were much more resistant to the inhibitor. These results suggest that translation of true late-gene transcripts is particularly sensitive to conditions of limited access to translation factors and that vhs is able either to prevent the limiting conditions or to facilitate translation initiation under these conditions. The varied permissivity of cell lines to vhs-null infection may stem from differences in the resilience of the translation machinery or the ability to control the accumulation of mRNAs.

Herpes simplex virus 1 (HSV-1), a large, enveloped DNA virus, is a master in subverting host cell functions for its own benefit. In particular, HSV-1 employs several mechanisms to block expression of cellular proteins and facilitate efficient synthesis of viral proteins (reviewed in references 29 and 55). The viral protein that executes the earliest attack on cellular gene expression is the virion host shutoff (vhs) protein (reviewed in reference 54).

The vhs protein, encoded by the UL41 gene, is part of the tegument and as such is delivered into the cytoplasm after fusion of the virion envelope with the host cell membrane (43). It is an endoribonuclease that degrades both cellular and viral mRNA (24, 32). The degradation of cellular mRNA presumably improves the access of viral mRNAs to the cellular translation machinery. The vhs-mediated increased turnover of viral mRNAs is thought to sharpen the transition between the successive expression of viral immediate-early (IE), early (E), and late (L) genes (32).

vhs is able to degrade all types of RNA in an *in vitro* system (10, 60, 61, 63); however, in infected cells vhs degrades only mRNA and spares other RNA species (24, 32). In an effort to explain this mRNA specificity, vhs has been shown to associate with the translation initiation complex eIF4F, which binds the

5' cap structure of mRNAs (33). In particular, vhs interacts with the eIF4F component eIF4AII, an ATP-dependent RNA helicase and the helicase cofactors eIF4H and eIF4B (7, 11). These observations suggest that interactions with eIF4AII, eIF4H, and eIF4B target vhs to actively translating mRNAs. This model is underscored by the finding that knockdown of eIF4H prevented vhs-mediated mRNA decay in cell culture (47).

vhs-mediated shutoff of host protein synthesis occurs mainly in the immediate-early and early phases of infection. Later in infection, the nuclease activity of newly synthesized vhs appears to be dampened by the viral proteins VP16 and VP22 (22, 25, 48, 52). This is thought to be necessary to ensure sufficient accumulation of viral late-gene transcripts and efficient virus production.

Since vhs destabilizes most mRNAs, it reduces synthesis of proteins involved in the innate and adaptive immune responses. This vhs-mediated downregulation is most likely the reason for the capacity of vhs to dampen the type I interferon (IFN) system (8, 31, 35), block activation of dendritic cells (45), and reduce production of proinflammatory cytokines and chemokines (58). These factors might account at least in part for the crucial role of vhs in HSV-1 virulence and pathogenicity (reviewed in reference 54). For example, vhs mutants are strongly attenuated in the corneas and central nervous system of mice (26, 34, 36, 56, 57). Accordingly, replication and virulence of vhs mutants is enhanced in knockout mice lacking IFN receptors (8, 26) or STAT1 (34, 36). However, even in IFN- $\alpha\beta\gamma^{-/-}$ and STAT $^{-/-}$ mice, the vhs mutant is still attenuated relative to the wild-type (WT) virus (26, 34, 36), suggesting that

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vhs contributes to efficient replication beyond its role as modulator of the immune response.

In cell culture, the attenuation of vhs-deficient HSV-1 is less pronounced than in mice. Similar to what was observed in the mouse model, the reduced virus yield in mouse embryonic fibroblasts (MEFs) is not completely due to IFN signaling, since virus titers of a vhs mutant in multicycle growth curve analyses were only partially restored in IFN- $\alpha\beta\gamma$ R^{-/-} MEFs (35). Knockout of the double-stranded-RNA (dsRNA)-dependent protein kinase R (PKR) protein did not alleviate this defect (35). vhs-deficient HSV-1 is also slightly attenuated in single-step growth curves in Vero cells, which lack IFN- α and IFN- β genes (39, 40, 53), suggesting an IFN-independent growth defect. The longstanding hypothesis advanced to explain this inherent growth defect is that the inappropriate overaccumulation of IE and E mRNAs might impair replication (32), but the exact mechanism by which this impairment might be achieved has not been thoroughly examined so far.

A potential clue to resolving this question came from two publications that point to a novel role for vhs in modulating translation. First, our group showed that vhs modulates translation in a bicistronic reporter gene assay without significantly altering the level of the respective mRNA (44). While expression of the 5' cistron was inhibited by vhs, expression of the 3' cistron was dependent on the sequence of the intergenic region. Expression driven by the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) was suppressed, whereas a mutant EMCV IRES and the cellular ApaF1, BiP, and DAP5 IRES elements facilitated increased expression in the presence of vhs. Interestingly, the 5' untranslated region (UTR) of the HSV-1 mRNAs for UL12, thymidine kinase (TK), or glycoprotein C (gC) also mediated vhs-dependent expression of the 3' cistron, suggesting that vhs may be involved in boosting translation of viral mRNAs during infection (44). Second, Esclatine and colleagues reported that vhs-deficient HSV-1 (F) induces formation stress granules (SGs) (9), which are considered diagnostic for the accumulation of stalled translation initiation intermediates (20, 21). SGs form as a response to various forms of stress (reviewed in reference 1). Depending on the type of stress, the serine/threonine kinases PKR, PKR-like endoplasmic reticulum kinase (PERK), general control nonrepressed 2 (GCN2), and heme-regulated inhibitor (HRI) are activated and phosphorylate the translation initiation factor eIF2 α (reviewed in reference 46). This event stalls the initiation process at the point of loading the initiator tRNA^{Met} onto the small ribosomal subunit (reviewed in reference 16) and results in accumulation of 48S preinitiation complexes containing mRNA, the translation initiation complex eIF4F, poly(A) binding protein (PABP), eIF3, eIF1, eIF1A, and the small ribosomal subunit (19). The preinitiation complexes are then assembled into SGs by proteins such as TIA-1 and TIAR (20, 21). Notably, SG formation and inhibition of translation can also occur independent of eIF2 α phosphorylation, for example, by blocking the activity of eIF4A with the novel compounds hippuristanol and pateamine (2, 3, 28). Since SGs are dynamic structures, mRNAs shuttle between SGs and polysomes to resume translation or are targeted to processing bodies for degradation (reviewed in reference 1).

Based on these observations, we hypothesized that inactivation of vhs may impair translation of viral proteins accounting

for the reduced virus yield. To test this hypothesis, we examined the effect of vhs mutations on SG formation, viral replication, and viral gene expression in various cell types. We report here that infection with vhs-deficient HSV-1 led to SG formation, reduced virus yield, and decreased accumulation of true late-gene products in HeLa cells and several additional cell lines but not in Vero cells. Furthermore, we determined that the reduced late-gene expression was due to reduced translational activity on true late-gene transcripts in HeLa cells. This block in translation initiation was specific for viral transcripts, i.e., cellular mRNAs were not affected. Inhibitor studies implied that the resilience of the translational machinery of the respective cell type might be critical for translation of true late-gene transcript in the absence of vhs.

Taken together, these findings suggest that in many cell types vhs plays an important role in boosting translation of late-gene products and that the lack of this activity might account for the inherent growth defect of vhs mutants.

MATERIALS AND METHODS

Cells and viruses. HeLa (cervical carcinoma), Vero (African green monkey kidney), U2OS (osteosarcoma), HEP2, and SK-N-SH (neuroblastoma) cells and HFF TEL 12 (telomerase immortalized human foreskin fibroblasts; kind gift from W. Bresnahan) were maintained in Dulbecco's modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human embryo lung (HEL) fibroblasts were cultured as described above except for the addition of 1 mM sodium pyruvate. The wild-type HSV-1 strains used in this study were KOS and its plaque-purified derivative KOS1.1 (17), and the vhs mutants were KOS-derived Δ Sma, which contains a 588-nucleotide deletion in the UL41 gene (40), vhs1, which contains a point mutation in the UL41 gene (39), and GFP vhs⁻, in which all but the last 7 codons of the UL41 open reading frame (ORF) are deleted and replaced by green fluorescent protein (GFP), so that GFP is under the control of the vhs promoter (4). Viruses were propagated, and the titer in Vero cells was determined. Virus absorption, infection, and mock infection were carried out at 37°C at a multiplicity of 10 PFU/cell.

Drug treatments. Phosphonoacetic acid (PAA; Sigma) was used at 300 μ g/ml starting at 1 h postinfection (hpi) to inhibit viral DNA replication. Thapsigargin (Sigma) was used at 1 μ M for 1 h prior to fixing to induce endoplasmic reticulum stress. Hippuristanol (a polyoxygenated steroid isolated from the coral *Isis hippuris* [3]) in dimethyl sulfoxide (DMSO) was used at concentrations ranging from 20 nM to 4,000 nM starting at 1 hpi to inhibit eIF4A activity. Corresponding amounts of DMSO were used as controls.

Immunofluorescence (IF) microscopy. Cells grown on coverslips were mock infected or infected with the respective viruses at a multiplicity of infection (MOI) of 10. At the indicated time points, cells were washed with phosphate-buffered saline (PBS), fixed with 3.65% paraformaldehyde for 15 min, permeabilized with ice-cold methanol for 10 min, washed with PBS, and blocked with 5% fetal bovine serum (FBS) or 2.5% human serum. Proteins were stained with primary antibodies specific for HSV-1 ICP4 (mouse, P1101; Virusys Corporation) and cellular TIA-1 (goat, C-20; Santa Cruz), TIAR (goat, C-18; Santa Cruz), PABP (mouse, 10E10; Santa Cruz), G3BP (mouse, 611126; BD Transduction Laboratories), and eIF4A1 (rabbit, ab31217; Abcam). Primary antibodies were detected by suitable secondary antibodies coupled to Alexa 488, Alexa 555, or Alexa 647 (Invitrogen). Images were obtained using an Axiovert 200 M fluorescence microscope, an ApoTome optical sectioning device (Zeiss), and the AxioVision 4.5 program (Zeiss). All image processing was performed using Photoshop CS3 (Adobe).

Western blot analysis. Mock-infected and infected cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% Igepal, 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% SDS, 0.25% sodium deoxycholate, 1 $\times$ Roche protease inhibitor, 10 mM Na- β -glycerophosphate, 2 mM Na₃VO₄, 1 mM NaF). Cleared lysates were analyzed by 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Hybond ECL; Amersham), and immunoblotted with primary antibodies specific for the HSV-1 proteins gC (mouse, P1104; Virusys Corporation), gB (mouse, P1123; Virusys), ICP4 (mouse, P1101; Virusys Corporation), ICP27 (mouse, P1113; Virusys Corporation), ICP34.5 (rabbit; kind gift from Ian Mohr),

US11 (mouse; kind gift from Bernard Roizman), UL47 (rabbit; kind gift from Gillian Elliott), thymidine kinase (TK) (rabbit; kind gift from William C. Summers), and VP16 (mouse, LP1; kind gift from Tony Minson) and the cellular proteins β -actin (mouse, A5441; Sigma), phospho-eIF2 α (rabbit, 9721; Cell Signaling), and eIF2 α (rabbit, 9722; Cell Signaling). Primary antibodies were detected by suitable secondary horseradish peroxidase (HRP)-conjugated antibodies using an enhanced chemiluminescence protocol (GE Healthcare) (see Fig. 3 and 7) or suitable secondary antibodies coupled to Alexa Fluor 680 (Invitrogen) or IRDye800 (Rockland), using the Odyssey infrared imaging system (LI-COR) (see Fig. S2 in the supplemental material).

RNA extraction and Northern blot analysis. Total RNA was isolated from cells in 60-mm culture dishes by use of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples (10 μ g) were subjected to 1.2% agarose-formaldehyde gel electrophoresis, visualized by SYBR gold staining, and transferred to a Genescreen membrane (NEN). Hybridizations of gC, US11, and p56 probes, radiolabeled with 32 P by random priming, were performed using ExpressHyb (Clontech) according to the user's manual. The gC and US11 probes were generated by PCR amplification of viral DNA using the primers 5' CCC AAACCAAGAACAACAC and 5' TGTTTCGTCAGGACCTCCTCT (gC) or 5' GGCGACCCAGATGGTTACTT and 5' GCGAGCCGTCAGTGGTTC (US11). The p56 probe has previously been described (30). γ -Actin transcripts were detected with a 32 P-labeled oligonucleotide, 5' AATGACCAGCGCGC GATCTCTTCTTCCATT. Hybridization was carried out in modified Westnat solution (6.6% SDS, 250 mM MOPS [morpholinepropanesulfonic acid] [pH 7.0], 5 $\times$ Denhardt's solution, 1 mM EDTA) at 53°C. Signals were analyzed with a Fujifilm FLA-5100 phosphorimager and Image Gauge version 4.22.

Polysome gradient. HeLa or Vero cells from two 100-mm culture dishes were mock infected or infected with KOS1.1 or Δ Sma at an MOI of 10. Cells were lysed at 12 hpi in lysis buffer (250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 50 mM Tris-HCl [pH 7.4], 0.5% Triton X-100, 100 μ g/ml cycloheximide, RNaseOUT). Cleared supernatant was incubated with MgCl₂ (final concentration, 10 mM) or EDTA (final concentration, 25 mM) for 10 min on ice. Lysates were layered onto gradients of 10 to 50% sucrose solution in 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 1.5 mM MgCl₂, 100 μ g/ml cycloheximide, 2 mM dithiothreitol (DTT), and RNaseOUT and centrifuged in an SW40 rotor for 105 min at 38,000 rpm and 4°C. Twenty fractions were collected from the top of the gradient, and their absorbance at 254 nm was measured. Samples were proteinase K treated, and RNA was phenol-chloroform extracted. Half of each RNA sample was subjected to Northern blot analysis as described above to determine the distribution of selected RNA species across the gradient.

RESULTS

Formation of stress granules in response to infection with vhs-deficient HSV-1 is cell type dependent. Esclatine et al. showed that infection of HEP-2 and HFF cells with the Δ U_L41 mutant virus but not wild-type HSV-1(F) led to the formation of cytoplasmic structures that stained positive for the SG marker antigens TIA-1/TIAR (9). We asked whether these cytoplasmic structures are true SGs and whether SG formation is a universal response to vhs-deficient HSV-1. Therefore, we infected cells with wild-type and vhs-deficient HSV-1 at an MOI of 10. Indirect immunofluorescence microscopy using antibodies against TIA-1 showed that Δ Sma but not KOS1.1 infection induced formation of cytoplasmic granules in HeLa cells at 16 h postinfection (hpi) (Fig. 1A). As expected, at late time points postinfection ICP4 localized to viral replication compartments and DAPI (4',6-diamidino-2-phenylindole) staining demonstrated chromatin marginalization. The cytoplasmic structures observed in Δ Sma-infected HeLa cells also stained positive for a second key SG marker, TIAR (see Fig. S1a in the supplemental material). Furthermore, immunostaining against TIA-1 or TIAR demonstrated that the cytoplasmic structures were also induced in response to the additional vhs mutants vhs1 and GFPvhs⁻ (Fig. S1B and C) but not in response to parental KOS (Fig. S1D), confirming that the observed phenotype is due to the loss of vhs function and not

due to other derivative-specific differences. Costaining of Δ Sma-infected HeLa cells with TIA-1/G3BP, G3BP/eIF4A, and PABP/eIF4A (Fig. 1B) revealed that the cytoplasmic structures contain G3BP, eIF4A, and PABP and hence are *bona fide* SGs. We also observed SG formation in Δ Sma-infected HEP-2, SK-N-SH, HFF, U2OS, and HEL cells (Fig. 1C). However, SG formation was less prominent in U2OS and HEL cells than in HeLa, HEP-2, and SK-N-SH cells, and HFF cells showed an intermediate phenotype. Surprisingly, however, we were not able to detect any SGs in Δ Sma-infected Vero cells (Fig. 1D). Although the TIA-1 signal in KOS1.1-infected Vero cells appeared somewhat speckled, these structures were mainly found in the nucleus and the cytoplasmic TIA signal was mostly dispersed. Therefore, this pattern clearly differed from SGs as seen in Δ Sma-infected HeLa cells. We considered the possibility that Vero cells are inherently unable to form SGs. However, endoplasmic reticulum stress induced by thapsigargin led to strong SG formation (Fig. 1D). This experiment verified that Vero cells are capable of forming SGs and suggests that Vero cells may have either different mechanisms to deal with the stress induced by vhs-deficient HSV-1 or the level of stress may be below the specific threshold to induce SG formation. One distinguishing feature of Vero cells is that they do not express IFN- α / β (6). However, IFN- α / β treatment did not induce SGs in HeLa cells (data not shown), making it unlikely that IFN- α / β signaling is responsible for the difference between HeLa and Vero cells. As HeLa and Vero cells are two of the most widely used cell lines in HSV-1 research, we felt that the striking difference in SG formation in response to vhs-deficient HSV-1 warranted further investigation.

SGs form late in infection and require viral DNA replication. To assess when SGs form during infection, we infected HeLa cells with Δ Sma at an MOI of 10 and stained for TIA-1 and the viral protein ICP4 at various time points. SGs formed as early as 8 hpi (Fig. 2A). The fraction of ICP4-positive SG-containing cells increased over time to greater than 50% at 16 hpi (Fig. 2A), suggesting that SG formation is triggered during the phase of late-gene expression. To further test this hypothesis, we treated Δ Sma-infected HeLa cells with phosphonoacetic acid (PAA), which inhibits viral DNA replication and consequentially transcription of true late genes. This treatment efficiently blocked formation of SGs (Fig. 2B), indicating that one or more events linked to viral DNA replication, such as accumulation of viral true late-gene transcripts, triggers SG formation.

Attenuated virus production in the absence of vhs is cell type dependent. In order to test whether the difference in SG formation in response to Δ Sma is reflected in differences in viral replication between HeLa and Vero cells, we performed a single cycle growth curve analysis. HeLa and Vero cells were infected with HSV-1 KOS1.1 or Δ Sma at an MOI of 10, and virus titers were determined at 1, 6, 12, 18, and 24 hpi. While KOS1.1 and Δ Sma replicated to similar titers in Vero cells, Δ Sma virus titers were reduced about 50-fold compared to the wild-type virus in HeLa cells (Fig. 3A). In HEP-2, HFF, and SK-N-SH cells, Δ Sma showed a growth phenotype similar to that observed in HeLa cells, with a difference in viral titers of vhs-deficient and wild-type virus between 29- and 45-fold (Fig.

FIG. 1. Characterization of stress granule (SG) formation in response to infection with wild-type or vhs-deficient HSV-1. (A) HeLa cells were mock treated or infected with HSV-1 KOS1.1 or ΔSma at an MOI of 10. At 16 hours postinfection (hpi), cells were stained for ICP4 (pink), TIA-1 (green), and nuclear DNA (blue), and the images in the lower row show the TIA-1 signal in white. (B) Costaining of ΔSma-infected HeLa cells against several SG marker proteins. HeLa cells were infected with ΔSma at an MOI of 10. At 16 hpi, cells were stained for TIA-1 (green) and G3BP (red), G3BP (green) and eIF4A (red), or PABP (green) and eIF4A (red). (C) HEP-2, SK-N-SH, HFF, U2OS, and HEL cells were infected with HSV-1 ΔSma and stained as described for panel A. (D) Vero cells were mock treated, infected with HSV-1 KOS1.1 or ΔSma at an MOI of 10, or treated with 1 μM thapsigargin for 1 h. At 16 hours postinfection (hpi), cells were stained as described for panel A.

3B), whereas U2OS and HEL cells showed a phenotype similar to that of Vero cells, supporting ΔSma virus replication to titers about 2- or 6-fold lower than those for KOS1.1, respectively (Fig. 3C). We are confident that during this single-step

replication cycle, the impact of IFN signaling was negligible, as demonstrated by the low to nondetectable mRNA levels of IFN-stimulated gene 56 under the conditions used as described below (see Fig. 5).

FIG. 2. (A) Dependency of SGs formation on the stage of infection. HeLa cells were infected with HSV-1 Δ Sma at an MOI of 10, fixed at the indicated time points postinfection, and stained for TIA-1 and ICP4. The percentage of cells containing SGs was calculated by counting ca. 300 ICP4-positive cells in several different fields. (B) HeLa cells were infected with HSV-1 Δ Sma at an MOI of 10 and mock treated or treated with 300 μ g/ml phosphonoacetic acid (PAA) starting at 1 hpi. At 16 hpi, cells were stained for TIA-1 (green), ICP4 (pink), and DNA (blue).

vhs is necessary for efficient accumulation of true late gene products in HeLa and other restrictive cell lines but not in Vero and other permissive cells. As vhs-deficient HSV-1 induces formation of SGs and formation of SGs is indicative of stalled translation initiation (reviewed in reference 1), we hypothesized that translation of viral transcripts might be reduced in the absence of vhs. To test this hypothesis, we examined lysates of infected HeLa or Vero cells by Western blot analysis. Detection of the viral proteins ICP4 (immediate-early), thymidine kinase (TK; early), and ICP34.5 (leaky late) showed that these proteins are expressed equally well in HeLa and Vero cells infected with KOS1.1 or the vhs mutant Δ Sma, GFPvhs⁻, or vhs1 at 6 and 12 hpi (Fig. 4A). The amounts of the leaky-late gene products VP16 and glycoprotein B (gB) were slightly reduced in the absence of vhs in HeLa cells but not in Vero cells (Fig. 4A). The true late-gene products glycoprotein C (gC) and US11, however, were strongly reduced in Δ Sma-infected HeLa cells compared to the level in KOS1.1-infected HeLa cells, while no difference in gC and US11 protein levels was observed in Vero cells (Fig. 4A). Lower gC and US11 protein levels in Δ Sma-infected HeLa cells were not due to differences in the accumulation of the respective mRNAs, as shown by Northern blot analysis of RNA extracted from HeLa and Vero cells infected with KOS1.1 or Δ Sma for 6 or 12 h (Fig. 4C). Given that both transcripts accumulate with true late kinetics, this observation furthermore excluded a potential defect in DNA replication of the vhs mutant. Reduced expression was also observed for another true late-gene product, UL47 (see Fig. S2 in the supplemental material). Figure S2 also shows that KOS displays the same phenotype as its plaque-purified derivative KOS1.1. Further Western blot analyses demonstrated that HFF, SK-N-SH, and HEP2 cells show a HeLa-like expression profile, with reduced expression of gC and US11 in the absence of vhs (Fig. 4B), whereas late-gene expression in U2OS and HEL cells was more similar to that in Vero cells, although the level of gC but not US11 protein was slightly reduced in U2OS cells (Fig. 4B). We also looked at ICP27 levels since gC expression strictly depends on ICP27 (38, 50). However, ICP27 was present in equal amounts in all samples (Fig. S2).

eIF2 α phosphorylation and p56 induction do not correlate with reduced late-gene expression. Phosphorylation of the translation initiation factor eIF2 α is a common cause for reduced protein expression in virus-infected cells and has been shown to be responsible for reduced late-gene expression with vhs-deficient HSV-2 (62). However, we found that eIF2 α is phosphorylated to similar extents in the presence and absence of vhs in HeLa cells (Fig. 4A). Consistent with this observation, expression of ICP34.5, a viral cofactor of eIF2 α dephosphorylation (14, 15), does not depend on the presence of vhs (Fig. 4A). It is possible that phosphorylation of eIF2 α in Δ Sma-infected U2OS and HFF cells contributes to the reduced expression of gC (Fig. 4B), despite efficient expression of ICP34.5 (data not shown). However, comparison of Western blot results from all cell types tested determined that the level of eIF2 α phosphorylation did not correlate with reduced late-gene expression in HeLa, SK-N-SH, and Hep2 cells. We also tested a possible involvement of p56 (ISG56, or IFIT1) in the reduced late-gene expression during vhs-null infection. ISG56 can be induced directly by virus infection or by IFN- α / β signaling and has been shown to interfere with translation by binding to the translation initiation factor eIF3 (12, 18). RNA from cells treated with 1,000 U IFN- α , mock treated, or infected with KOS1.1 or Δ Sma at an MOI of 10 was extracted at 6 and 12 hpi and analyzed by Northern blotting with probes specific for p56 and γ -actin (Fig. 5). As expected, γ -actin mRNA levels were reduced in the presence of wild-type vhs. Compared to the IFN- α treated cells, only a weak signal for p56 mRNA was detected in Δ Sma-infected HeLa and Hep2 cells, whereas no p56 mRNA was detected in all other cell types tested. It is not clear whether these low levels of p56 affect translation, and we cannot rule out that p56 contributes slightly to the phenotype in HeLa and Hep2 cells. However, similar to eIF2 α phosphorylation, p56 induction was not found to strictly correlate to the reduced late-gene expression phenotype when results from all cell types were compared.

vhs increases translation of late-gene transcripts in HeLa cells. The experimental data gathered so far support the hypothesis that late-gene expression is blocked at the level of translation. Therefore, we directly assessed the translational

FIG. 3. Single-cycle growth curve analyses. HeLa and Vero cells (A), HEp2, HFF, and SK-N-SH cells (B), or HEL and U2OS cells (C) were infected with KOS1.1 or Δ Sma at an MOI of 10. At the indicated time points, cells and supernatants were harvested. Viral titers were determined by a plaque assay for Vero cells. The graphs represent the averages of results from three independent experiments. Error bars indicate the standard deviations.

activity on gC and US11 mRNAs. We mock treated or infected HeLa or Vero cells with KOS1.1 or Δ Sma, prepared lysates at 12 hpi, and analyzed polysome profiles by sucrose density centrifugation. As a control, we also examined the sedimentation profiles of lysates that were treated with EDTA to dissociate the polysomes. RNA was extracted from fractions 1 to 20 (top to bottom) and subjected to agarose gel electrophoresis. Stain-

FIG. 4. Analyses of protein profiles in different cell types infected with wild-type and vhs mutant HSV-1. (A) HeLa or Vero cells were mock treated or infected with KOS1.1 or the vhs mutant Δ Sma, GFPvhs⁻, or vhs1. Lysates were prepared at 6 and 12 hpi and analyzed by immunoblotting with antibodies specific for gC, US11, phospho-eIF2 α , total eIF2 α , β -actin, gB, VP16, ICP34.5, TK, and ICP4. (B) HFF, SK-N-SH, HEp2, HEL, and U2OS cells were mock treated or infected with KOS1.1 or Δ Sma. Lysates were prepared 6 and 12 hpi and analyzed by immunoblotting with antibodies specific for gC, US11, phospho-eIF2 α , eIF2 α , and ICP4. (C) HeLa or Vero cells were mock treated or infected with KOS1.1 or Δ Sma. RNA was extracted at 6 and 12 hpi and analyzed by Northern blot with probes specific for gC or US11 mRNA.

FIG. 5. Induction of p56 mRNA. Vero, HeLa, HEp2, SK-N-SH, HFF, U2OS, and HEL cells were mock treated, infected with KOS1.1 or Δ Sma at an MOI of 10, or treated with 1,000 U IFN- α 6 h prior to harvest. RNA was extracted at 6 and 12 hpi and analyzed by Northern blotting with probes specific for p56 and γ -actin mRNA.

ing of rRNA with SYBR gold showed that 40S ribosomal subunits are found in fractions 3 and 4, 60S-80S complexes are found in fractions 5 to 7, and higher fractions represent polysomes (see Fig. S3A and S4A in the supplemental material).

The RNA was further analyzed by Northern blotting with probes specific for gC and US11 mRNA and cellular γ -actin mRNA and detected by phosphorimaging (Fig. S3B, C, and D and S4B, C, and D). The distribution of the respective mRNA signal over the gradients is graphically represented in Fig. 6 as the percentage of total mRNA signal present in each fraction. As expected from the Western blot data, viral gC and US11 mRNA from both KOS1.1 and Δ Sma-infected Vero cells sedimented in the fractions representing polysomes, indicating efficient translation initiation (Fig. 6D and E). Although the peak for gC mRNA from Δ Sma-infected Vero cells was slightly shifted toward smaller fractions, the majority of the mRNA was still in the polysome fraction. The gC and US11 mRNA from KOS1.1-infected HeLa cells was also mainly found in polysome fractions, although with more of the mRNA signal at lower fractions than for KOS1.1-infected Vero cells (Fig. 6A and B). In contrast, gC and US11 mRNA from Δ Sma-infected HeLa cells was shifted toward fractions representing monosomes or preinitiation complexes and overlapped with mRNAs from EDTA-dissociated polysomes (Fig. 6A and B). This result indicated that in the absence of vhs in HeLa cells, translation initiation on viral true late transcripts is strongly compromised, whereas vhs has little impact on translation efficiency in Vero cells.

Reduced translation is specific for viral transcripts. Interestingly, vhs is not required for the translation of cellular γ -actin mRNA, as the majority of the mRNA was found in polysome fractions in both mock-treated and Δ Sma-infected HeLa

FIG. 6. Polysome analyses. HeLa cells (A, B, C) or Vero cells (D, E, F) were mock treated or infected with KOS1.1 or Δ Sma at an MOI of 10. Cells were lysed 12 hpi, incubated with or without EDTA, and analyzed by sedimentation through a 10 to 50% sucrose gradient. Samples were taken from top to bottom, and extracted RNA was analyzed by Northern blotting for the viral mRNAs for gC (A, D) and US11 (B, E) or the cellular γ -actin mRNA (C, F). Bands were quantified by a phosphorimager, and the distribution of the mRNAs across the gradient is shown as a percentage of the total signal of the respective mRNA. The graphs represent one out of three comparable experiments.

FIG. 7. Metabolic labeling of proteins in virus-infected HeLa or Vero cells. Cells were either mock infected or infected with KOS1.1 or Δ Sma at an MOI of 10. At 5 or 11 hpi, cells were metabolically labeled with [35 S]methionine for 1 h. Cell extracts were prepared and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The positions of the molecular mass markers are indicated on the left. (●, cellular protein; ▲, viral protein).

and Vero cells (Fig. 6C and F). This finding was corroborated by metabolic labeling of infected cells (Fig. 7). HeLa cells and Vero cells were mock treated or infected with KOS1.1 or Δ Sma at an MOI of 10 and labeled with [35 S]methionine at 5 or 11 hpi. Lysates were prepared at 6 or 12 hpi and subjected to PAGE and autoradiography. In KOS1.1-infected HeLa cells, virus-specific protein bands were clearly visible at 6 and 12 hpi, whereas translation of cellular proteins was strongly

reduced (Fig. 7). In Δ Sma-infected HeLa cells, some virus-specific signals were weaker or not detectable at 12 hpi (Fig. 7, arrowheads), while cell specific bands were as strong as in mock-infected cells (Fig. 7, dots). In contrast, the translation patterns of KOS1.1 and Δ Sma-infected Vero cells showed only minor differences (Fig. 7).

Viral late-gene expression in Vero cells is more resistant to an eIF4A inhibitor than that in HeLa cells. As demonstrated above, the SG formation and reduced late-gene expression observed during vhs-null infection do not correlate with enhanced eIF2 α phosphorylation (Fig. 3). However, it was recently demonstrated that SGs can be induced independently of eIF2 α phosphorylation by antagonizing the function of the RNA helicase eIF4A using inhibitors such as hippuristanol (3, 28). This observation, and the reported interactions of vhs with eIF4A and its cofactors eIF4B and eIF4H, raised the possibility that eIF4A activity is impaired in the absence of vhs, leading to the observed defect in late-gene expression. If so, then one might predict that hippuristanol treatment would mimic a vhs-null phenotype in KOS1.1-infected HeLa and Vero cells. Indirect immunofluorescence microscopy confirmed that treatment of uninfected HeLa and Vero cells with hippuristanol led to formation of SGs in both cell types, as expected. However, HeLa cells were much more sensitive than Vero cells, forming SGs in response to 300 nM hippuristanol, compared to 4,000 nM in Vero cells (Fig. 8A, top row, and B, top row). In addition, treatment of HeLa cells with as little as 80 nM hippuristanol sensitized cells so that infection with KOS1.1 resulted in formation of SGs (Fig. 8A, bottom row), whereas in Vero cells 2,500 nM hippuristanol was needed to drive cells to form SGs in response to KOS1.1 and Δ Sma virus infection (Fig. 8B, middle and bottom rows). In accordance with the IF results, we found a prominent difference in how hippuristanol treatment affected translation of true late mRNAs in HeLa and Vero cells. Western blot analysis of cells infected with KOS1.1 at an

FIG. 8. Impact of hippuristanol on SG formation in HeLa and Vero cells. HeLa cells (A) or Vero cells (B) were mock treated or infected with KOS1.1 and Δ Sma at an MOI of 10. Hippuristanol was added at 1 hpi. At 16 hpi, cells were stained for TIA-1 (green), ICP4 (pink), and DNA (DAPI; blue). Images shown represent the largest amount of hippuristanol without formation of SGs, the smallest amount of hippuristanol inducing SGs, and a larger amount of hippuristanol showing dose-dependent increase of SG formation.

FIG. 9. Impact of hippuristanol treatment on expression of viral proteins in HeLa and Vero cells. HeLa cells (A) and Vero cells (B) were mock treated or infected with KOS1.1 or Δ Sma at an MOI of 10. At 1 hpi, cells were treated with hippuristanol as indicated or treated with DMSO as a control. Lysates were prepared at 12 hpi and analyzed by immunoblotting with antibodies specific for gC, US11, ICP34.5, ICP4, and β -actin. (C) Hippuristanol treatment does not reduce the level of gC mRNA. HeLa cells were mock treated or infected with KOS1.1 or Δ Sma at an MOI of 10. At 1 hpi, cells were treated with hippuristanol as indicated or treated with DMSO as a control. RNA was extracted at 12 hpi and analyzed by Northern blotting for the viral gC mRNA or the cellular γ -actin mRNA.

MOI of 10 for 12 h showed that in HeLa cells as little as 20 nM hippuristanol decreased gC and US11 levels and 80 nM hippuristanol blocked accumulation of these proteins (Fig. 9A). At these concentrations, hippuristanol had only minor effects on accumulation of ICP34.5 and ICP4 and did not affect actin levels (Fig. 9A). Northern blot analysis confirmed that hippuristanol treatment did not affect gC or US11 mRNA levels (Fig. 9C), indicating that the reduced protein expression is due to the effect of hippuristanol on translation and not on transcription or mRNA stability. Furthermore, it has been reported that hippuristanol does not induce phosphorylation of eIF2 α (28). Similar to what was observed in the induction of SGs, far larger amounts of hippuristanol were required in Vero cells (1,280 nM) to significantly reduce gC and US11 levels during KOS1.1 and Δ Sma infection (Fig. 9B). These results suggest that translation in Vero cells is more resilient during conditions of reduced availability of translation factors than translation in HeLa cells. The apparently selective effect of low doses of hippuristanol on viral late- versus IE- and E-gene

expression in HeLa cells further implies either that translation of late-gene transcripts is more sensitive to restrictive conditions than translation of IE and E mRNAs or that conditions become more restrictive during the late stages of infection.

DISCUSSION

It is well established that vhs degrades viral and cellular mRNAs during the early stages of infection. However, there is mounting evidence that vhs also plays a role in translation. First, vhs associates with the translation initiation factors eIF4A, eIF4B, and eIF4H (7, 11). Furthermore, we have previously shown that vhs increases translation of the second cistron driven by several IRES elements or viral 5' UTRs in a bicistronic reporter gene assay (44). Finally, infection with vhs-deficient HSV-1 leads to formation of TIA-1/TIAR-positive cytoplasmic foci, presumably SGs (9), which might indicate stalled translation initiation. In this study, we demonstrated that vhs boosts translation of viral late-gene products and promotes efficient virus production in a cell type-dependent manner and that the requirement for vhs might be determined by the resilience of the translation machinery in the respective cell type. This novel role of vhs during HSV-1 infection is supported by the following findings. (i) HSV-1 with mutated vhs or partial or complete deletion of the vhs ORF, but not wild-type viruses, triggered formation of SGs in all cell types tested except Vero cells. (ii) The vhs mutant Δ Sma was attenuated in several cell lines, including HeLa cells, but not in Vero, HEL, or U2OS cells. (iii) Expression of true late genes was strongly reduced in the absence of functional vhs in HeLa and other restrictive cells but not in Vero, HEL, or U2OS cells, showing a strong correlation with the results of the growth curve. (iv) Reduced gene expression in Δ Sma-infected HeLa cells was due to decreased translation initiation on the respective viral mRNAs. (v) This effect was specific for late viral transcripts since translation of cellular mRNAs was not affected in the absence of vhs. (vi) Treatment of HeLa and Vero cells with the eIF4A inhibitor hippuristanol induced SG formation and reduced late-gene expression during KOS1.1 infection, mimicking the phenotype of a Δ Sma infection. The effective dose for HeLa cells was far lower than that for Vero cells, indicating that the resilience of the translational machinery differs between HeLa and Vero cells. This difference might be the critical factor for translation of true late-gene transcript in the absence of vhs and might determine the scale of the inherent growth defect of vhs-deficient HSV-1.

It is not yet clear whether SG formation directly impedes virus production or, alternatively, whether the SGs are only a readout of the stalled translation initiation. TIA-1^{-/-} or TIAR^{-/-} MEFs have been shown to support replication of HSV-1 (strain 129) to higher titers than WT MEFs (27), indicating that TIA-1 and TIAR have a negative effect on viral replication. On the other hand, Wylie and coworkers showed that knockout of TIA-1 or TIAR in MEFs did not restore replication of an HSV-2 vhs mutant to wild-type titers (62). However, they did not determine whether the knockouts resulted in an increase in virus production relative to that in wild-type MEFs and, if so, whether this increase was different for HSV-2 and the vhs mutant. While it will be interesting to study the Δ Sma phenotype in the absence of TIA-1 or TIAR,

we speculate that SG formation is more likely a readout than a cause of the Δ Sma phenotype because the reduced gC and US11 protein levels are observed as early as 6 hpi, whereas SG formation starts between 8 and 10 hpi.

We suggest that the accumulation of high levels of viral late mRNAs following the onset of viral DNA replication overwhelms the capacity of the translational initiation machinery, leading to the observed translational defect in restrictive cells. The observation that hippuristanol generates a similar phenotype in cells infected with wild-type virus is consistent with this hypothesis and further suggests that eIF4A may be one of the limiting factors. In this context, it is interesting to note that the 5' UTRs of many HSV mRNAs adopt quite stable secondary structures because of the overall GC-rich nature of the HSV genome. For example, the 5' UTRs of ICP4, TK, ICP34.5, gC, and US11 mRNAs have predicted values of -125 , -31 , -103 , -63 , and -80 kcal/mole, respectively, as estimated by the Mfold algorithm. Most 5' UTRs of well-translated cellular mRNAs have little or no secondary structure, with predicted free energies between 0 and -30 kcal/mole (5), whereas secondary structures with a free energy of -50 kcal/mole or lower impose a strong block on ribosome scanning (23, 37). For highly structured 5' UTRs, optimal helicase activity is absolutely critical for initiation of translation (59). Thus, it seems quite plausible that eIF4A could become limiting due to the massive accumulation of highly structured viral mRNAs during the late stages of infection. As shown in Fig. 8 and 9, Vero cells can deal with over 10 times more hippuristanol than HeLa cells before the balance is tipped toward SG formation and a block in translation of viral late-gene transcripts. These observations suggest that translation in Vero cells is more resistant to reduced eIF4A availability than translation in HeLa cells. Therefore, vhs would be less critical for late-gene expression under these circumstances. It is also possible that Vero cells are more efficient at degrading excess mRNA to avoid overloading of the translation machinery.

Interestingly, the translational blockade observed in HeLa cells in the absence of vhs appears to selectively impair translation of viral true late mRNAs, sparing other mRNAs that are present in the infected cells. Thus, cellular γ -actin mRNA remains associated with large polysomes late during vhs-null infection (Fig. 6), and many other cellular and viral mRNAs are also translationally active as judged by [35 S]methionine incorporation (Fig. 7, 12-hpi lanes). How might this selective blockade arise? One possibility is that some structural feature of late mRNAs places them at a disadvantage relative to other mRNAs. Alternatively, perhaps true late transcripts fail to be efficiently translated simply because they accumulate after translation initiation factors have become limiting. According to the latter model, the limiting factors are stably sequestered by the preexisting cellular and viral mRNAs and are not readily recycled in the absence of vhs. Further studies are required to distinguish between these and other possibilities.

There are several possibilities for how vhs boosts translation initiation on true late mRNAs in restrictive cells. The simplest is that vhs prevents "mRNA overload" during the late stages of infection by eliminating host mRNAs and promoting the decay of viral IE and E transcripts. A second (and related) possibility is that vhs promotes recycling of translation initiation factors by shortening the half-life of viral mRNAs, allowing transfer of

factors from the viral IE, E, and leaky late mRNAs made before DNA replication onto true late mRNAs. A third possibility is that vhs plays a more active role beyond reducing mRNA levels and recycling translation initiation factors. eIF4B and eIF4H are cofactors of eIF4A that increase the helicase activity, and their requirement correlates with the degree of secondary structure in the 5' UTRs of mRNAs (41, 42, 51). vhs has been shown to interact with eIF4A, eIF4B, and eIF4H (7, 11). Therefore, we speculate that vhs might act as an adaptor to bring helicase and cofactors together and thereby increase unwinding of the secondary structure. vhs has no defined RNA-binding capacity, so how would it assemble helicase and cofactors preferably on true late-gene transcripts? Although we cannot yet answer this question, it is possible that vhs is targeted to mRNAs as a complex with VP16 and VP22, of which the latter has been shown to bind to mRNAs (49). Furthermore, late in infection the RNase activity of vhs would be dampened in a complex with VP16 and VP22 (13, 22, 25, 48, 52).

These three models for how vhs boosts translation of true late-gene transcripts are not mutually exclusive, and we are currently investigating the potential role of eIF4A availability, RNA accumulation, 5' UTR structure, and interaction of vhs with translation initiation factors.

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