Herpes Simplex Virus 2 Expresses a Novel Form of ICP34.5, a Major Viral Neurovirulence Factor, through Regulated Alternative Splicing

Shuang Tang, Nini Guo, Amita Patel, Philip R. Krause

Division of Viral Products, Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA

Herpes simplex virus 1 (HSV-1) and HSV-2, two closely related neurotropic human herpesviruses, achieve neurotropism through ICP34.5, a major viral neurovirulence factor. In this report, in addition to the full-length 38-kDa protein (ICP34.5 α), we identified a 28-kDa novel form of ICP34.5 (ICP34.5 β) in HSV-2-infected cells. ICP34.5 β is translated from unspliced ICP34.5 mRNA, with the retained intron introducing a premature stop codon. Thus, ICP34.5 β lacks the C-terminal conserved GADD34 domain but includes 19 additional amino acids encoded by the intron. Although a fraction of both HSV-2 ICP34.5 proteins are detected in the nucleolus, ICP34.5 α is predominantly located in cytoplasm, and ICP34.5 β is mainly detected more diffusely in the nucleus. ICP34.5 β is unable to counteract PKR-mediated eIF2 phosphorylation but does not interfere with ICP34.5 α 's function in this process. Efficient expression of ICP34.5 β in cell culture assays is dependent on viral infection or expression of ICP27, a multifunctional immediate-early gene. The effect of ICP27 on the ICP34.5 β protein level is attributed to its selective inhibition of ICP34.5 splicing, which results in increased expression of ICP34.5 β but a reduced level of ICP34.5 α . The C-terminal KH3 domain but not the RNA binding domain of ICP27 is required for its specific inhibition of ICP34.5 splicing and promotion of ICP34.5 β expression. Our results suggest that the expression of ICP34.5 α and ICP34.5 β is tightly regulated in HSV-2 and likely contributes to viral pathogenesis.

Herpes simplex virus 1 (HSV-1) and HSV-2 are closely related neurotropic herpesviruses. HSV-1 typically infects the facial region and establishes a lifelong latent infection in sensory neurons of the trigeminal ganglia, while HSV-2 typically infects the genital region and establishes a lifelong latent infection in sensory neurons of the sacral dorsal root ganglia. Periodically, either virus may reactivate to cause symptomatic or asymptomatic recurrences in the area served by these sensory neurons. ICP34.5, a major viral neurovirulence factor, is required for efficient viral replication in neurons (1–4). ICP34.5 deletion mutants show significantly decreased replication in the human brain (5–7). Although ICP34.5 is characterized as a γ 1 late gene (8), the ICP34.5 protein is detectable as early as 2 h postinfection in infected cell cultures (9). The ICP34.5 gene is located in the internal and terminal repeat region of the viral genome and is mapped antisense to the latency-associated transcripts (LATs) and the long/short junction transcript (L/ST). Previous studies suggested that ICP34.5 expression is regulated by several mechanisms. Overexpressing the L/ST transcript by mutating the ICP4 binding site at the transcriptional starting site of L/ST dramatically reduces ICP34.5 expression (10–12). In addition, two latently and acutely expressed HSV-2 LAT-encoded miRNAs target both the 5' untranslated region (UTR) and exon 1 of ICP34.5 (13–18). This regulated ICP34.5 expression may play an important role during establishment of viral latency and spontaneous reactivation.

The mechanism by which ICP34.5 promotes viral neurovirulence is not entirely clear. Both HSV-1 and HSV-2 ICP34.5 contain a conserved C-terminal GADD34 domain (19, 20) and a less conserved N-terminal domain. The C-terminal GADD34 domain of ICP34.5 counteracts protein kinase R (PKR)-mediated Ser-51 phosphorylation of eIF2, and therefore blocks translation shutoff by abolishing its interaction with protein phosphatase 1 (PP1) (19, 20). HSV-1 ICP34.5 mutants with deletion of the GADD34 domain are unable to replicate in neuronal cells and are not neurovirulent (2, 21). The N-terminal region of HSV-1 ICP34.5 binds to

Beclin-1 and inhibits autophagy, which contributes to neurovirulence in a PKR-dependent manner (22, 23). The N-terminal domain of HSV-1 ICP34.5, which partially overlaps the Beclin-1 binding domain (see Fig. 2A), also interacts with TANK-binding kinase 1 (TBK1) to disrupt interferon-regulatory factor 3 (IRF3) activity and beta interferon (IFN- β) expression (24, 25). Studies also suggested that the N-terminal domain of HSV-1 ICP34.5, overlapping with Beclin-1 and TBK1 binding domains (amino acids [aa] 30 to 106), is important for efficient virus egress/release in mouse cells (26, 27), although it is not clear whether this function is related to its interaction with Beclin-1 or TBK1.

HSV ICP27, an immediate-early (IE) gene, is highly conserved between HSV-1 and HSV-2. ICP27 is a multifunctional protein and plays an essential role in the expression of viral late genes (28, 29). ICP27 interacts and colocalizes with cellular splicing factors, including small nuclear ribonucleoproteins (snRNPs) (30), SR proteins, SR protein kinase 1 (SRPK1) (31), and spliceosome-associated protein 145 (SAP145) (32), and is suggested to inhibit the splicing of cellular pre-mRNAs by impairing spliceosomal assembly (for a review, see reference 33). Recently, HSV-1 ICP27 has been shown to modify alternative splicing of HSV-1 gC protein and promyelocytic leukemia (PML) isoforms (34, 35).

Although closely related, HSV-1 and HSV-2 are clearly different from each other in terms of viral pathogenesis. For example, HSV-2 is generally more neurovirulent than HSV-1 in various experimental animal models (36–38). Unlike its HSV-1 homologue, HSV-2's major viral neurovirulence factor, ICP34.5, is a

Received 20 December 2012 Accepted 5 March 2013

Published ahead of print 13 March 2013

Address correspondence to Philip R. Krause, philip.krause@fda.hhs.gov.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.03500-12

spliced gene and contains an intron (39). HSV-2 ICP34.5 also lacks the central proline-alanine-threonine (PAT) repeats, which are required for HSV-1 neuroinvasion and are also critical for the subcellular localization of HSV-1 ICP34.5 protein and for viral release (40–42). In addition, several key functions of HSV-1 ICP34.5 such as binding with TBK1 and Beclin-1 that are attributed to the less conserved N-terminal domain have not been verified in HSV-2 ICP34.5. Thus, more complete knowledge of the molecular function of HSV-2 ICP34.5 will improve understanding of viral neurovirulence and the differences between HSV-1 and HSV-2 pathogenesis. In this report, we identified a novel truncated form of ICP34.5 (ICP34.5 β) in HSV-2-infected cells. ICP34.5 β is encoded by an unspliced ICP34.5 mRNA and exhibits distinct function and subcellular localization compared to its spliced counterpart (ICP34.5 α). Efficient expression of ICP34.5 β is dependent on viral infection or ICP27 expression. Further, we showed the C-terminal domain of ICP27 is required for the efficient and specific inhibition of ICP34.5 splicing.

MATERIALS AND METHODS

Cells, viruses, and antibodies. HSV-2 strain HG52 (GenBank accession no. NC_001798) and HSV-1 strain 17syn+ (GenBank accession no. NC_001806) genomic sequences were used as reference sequences. ICP34.5 protein sequences were deduced by translating these nucleic acid sequences. Vero, 293, HeLa, and U2OS cell lines were obtained from ATCC. HSV-2 strain 333 was obtained from Gary Hayward (Johns Hopkins University, Baltimore, MD). HSV-1 ICP34.5 deletion mutant virus R3616 and its deletion-repaired rescuant virus, R3659, were obtained from Bernard Roizman (University of Chicago, Chicago, IL). Rabbit polyclonal anti-HSV-2 ICP34.5 and ICP0 were raised against synthetic peptides as reported previously (14, 15). Mouse monoclonal anti-HSV ICP27 antibody (Ab31631) was obtained from Abcam. Anti- β -tubulin was obtained from BD Bioscience. Antibodies for human Phospho-eIF2 α (Ser51) and eIF2 α were obtained from Cell Signaling Technology. Monoclonal mouse anti-C23 antibody (MS3) was obtained from Santa Cruz Biotech. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 555-conjugated goat anti-mouse IgG were obtained from Invitrogen.

Plasmids. Full-length HSV-2 ICP34.5 (pICP34.5-full) was cloned by first inserting a PCR-amplified HSV-2 ICP34.5 region, including the 5' UTR, the entire coding region and its stop codon using the PCR primers oST395 (GGAAAAGAGGCGGGGCGGGAGTCC) and oST426 (GGTTC AACCTAGACCGCCCGACGG) into a pCR4 Topo clone vector (Invitrogen) and then subcloning into the pFlag vector (Sigma) EcoRI site. pICP34.5 α was cloned by first inserting a reverse transcription-PCR (RT-PCR)-amplified spliced HSV-2 ICP34.5 region with the primers oST432 (GAGCCAGCCGCCCCCATGT) and oST426 (using cDNA from HSV-2 strain 333-infected Vero cell as a template) and then subcloning into the pFlag vector (not in frame with flag tag). pICP34.5 β was cloned by first inserting a PCR-amplified HSV-2 ICP34.5 region, including exon 1 and a partial intron that contains the stop codon with the primers oST432 and oST398 (GAGGGGCGTCAGGGGTCGGAGG) and then subcloning into the pFlag vector (not in frame with flag tag). pICP27 was cloned by inserting a PCR fragment containing the HSV-2 ICP27 coding region with the primers oST718 (CACCATGGCTACCGACATTGATAT GCTAATCGA) and oST719 (AAATAGGGAGTTGCAGTAGAAGTATT TGCC) into a pcDNA3.1 Topo directional clone vector (Invitrogen). HSV-2 ICP27 mutant plasmids, including d1-2 (with deletion of aa 12 to 63), Δ RGG (with deletion of the RGG motif from aa 133 to 155), Δ RR2 (with deletion of RGG and adjacent RNA binding sequence from aa 133 to 171), and M15 (with a two amino acid mutations, including Pro466Leu and Gly467Glu at the KH3 domain) were obtained from Masatoshi Hagiwara and Takayuki Nojima (Tokyo Medical and Dental University, Tokyo, Japan). HSV-1 ICP34.5 was amplified using oST760 (CACCATGGC CCGCCGCCGCCAT) and oST764 (GACCGAGTTCGCCGGGGC

GGCT) as primers and the HSV-1 strain 17 DNA as a template and cloned into the pcDNA3.1 Topo direction clone vector (Invitrogen). pST1 (a KSHV K8 expression plasmid) and pWX1 (an HPV16 E6E7 expression plasmid) were obtained from Zhiming Zheng (National Cancer Institute, Bethesda, MD) (43, 44).

Transfection, RT-PCR, and Western blot. Five million 293 cells were transfected with plasmids in six-well plates with plasmids indicated in the figures using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. cDNA was made using the first-strand cDNA kit (Invitrogen) using 2 μ g of total RNA prepared by the All-Prep DNA/RNA kit (Qiagen) from transfected or infected cells. RT-PCR using various cDNAs and the primers indicated in the figures were performed with a Hot-Star high-fidelity *Taq* polymerase kit (Qiagen). Oligonucleotides used in RT-PCRs to amplify specific genes as indicated in the figures included oST432, oST398, oST426, and oST731 (TTGTAGTCAGCCCGGGATCCTC), oST732 (GGAGAGCGGGCCTCGCGAGAC), oST728 (GACCTGTAC GCCAACACAG), oST729 (TCGTCATACTCTGCTTGC), oST724 (CATGGCAGCAGTAAAGCAAG), oST725 (GCATTGTTCCCATAGA GTTCC), oST726 (TACATGTTCCAATATGATT), oST727 (GTGGAC TCCACGACGTAATC), oST1 (ACCACCAAGAGGACCACACATTTC), oST3 (CACACAAAGTCTGGCATGGTTCTCCC), pr106 (GTTTCAGG ACCCAGAGGAGC), and pr562 (TGATTACAGCTGGGTTTC). Total proteins from infected or transfected cells were prepared in 2 $\times$ sodium dodecyl sulfate (SDS) sample buffer, separated by a NuPAGE 4 to 12% polyacrylamide gel electrophoresis (PAGE) gel (Invitrogen), and transferred to a nitrocellulose membrane (Invitrogen). Western blots were performed with corresponding antibodies on the same membranes after stripping.

Immunofluorescence assay for HSV-2 ICP34.5 in transfected and infected cells. Vero cells were grown on glass coverslips in six-well plates overnight before transfection with 0.5 μ g of pICP34.5 β , pICP34.5 α , or pICP34.5-full. Cells on the coverslips were washed twice with phosphate-buffered saline (PBS) and fixed with 1 $\times$ PBS containing 4% paraformaldehyde at 24 h posttransfection. Cells were permeabilized and blocked in a PBS solution containing 0.1% Triton X-100 and 10% normal goat serum (Sigma) for 1 h. The coverslips were incubated with a rabbit anti-HSV-2 antibody solution containing 0.1% Triton X-100, 10% normal goat serum, and 1:100 diluted anti-ICP34.5 antibody for 2 h and then washed for four times with a PBS wash solution containing 0.1% Triton X-100. After a washing step, the coverslips were incubated with fluorescein-conjugated goat anti-rabbit IgG (Vector Laboratories, CA) solution (containing 1 $\times$ PBS, 0.1% Triton X-100, 10% normal goat serum, and 1:50-diluted fluorescein-conjugated goat anti-rabbit IgG) for 1 h. The coverslips were then washed four times with the wash solution before being mounted with Fluoromount-G containing DAPI (Southern Biotech). The stained cells were examined using a Nikon ECLIPSE Ti Fluoresce microscope (Nikon, NY). Vero cells grown on four-well chamber slides (Fisher Scientific, MA) were infected with or without HSV-2 strain 333 at a multiplicity of infection (MOI) of 10. The cells were fixed at 5 or 16 h postinfection (hpi) and then stained with the anti-HSV-2 ICP34.5 antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG according to the procedures described above. The stained cells were washed and mounted with Fluoromount-G containing DAPI (4',6'-diamidino-2-phenylindole). The chamber slides were examined using a Zeiss laser scan confocal microscope LSM 510 (Carl Zeiss, Germany) with a $\times$ 63 oil objective. A pinhole was set at 1 airy unit (AU), and a $\times$ 1.5 zoom was used. Similarly, U2OS cells grown on four-well chamber slides (Fisher Scientific, MA) were transfected with pICP34.5 α or pICP34.5 β . At 24 h posttransfection, cells were fixed with methanol (-20°C) or 4% paraformaldehyde. The cells were washed three times with PBS and then permeabilized and blocked in PBS containing 0.1% Triton X-100 and 10% normal goat serum for 1 h before incubation with 1:100-diluted anti-ICP34.5 antibody and 1:50-diluted anti-C23 antibody overnight. After washing, the coverslips were incubated with a secondary antibody solution (containing 1 $\times$ PBS, 0.1% Triton X-100, 10% normal goat serum, 1:50-diluted Alexa

FIG 1 HSV-2 ICP 34.5 is alternatively spliced, resulting in the two protein products ICP34.5 α (38 kDa) and ICP34.5 β (28 kDa). (A) Two protein bands with sizes corresponding to 38 and 28 kDa were detected using a HSV-2 ICP34.5-specific antibody in Vero cells infected with HSV-2. Vero cells were infected with HSV-2 at a multiplicity of infection (MOI) of 2. Total cell proteins prepared at 0, 3, 5, 8, and 16 hpi were separated by a using a 4 to 12% PAGE gel and transferred to a nitrocellulose membrane. The same membrane was blotted with an anti-HSV-2 ICP34.5 antibody, an anti-HSV-2 ICP0 antibody, and an anti-tubulin antibody after stripping. The second panel is an overexposed image of the first panel. (B) HSV-2 ICP34.5 is not efficiently spliced in HSV-2-infected cell cultures. Total RNA from Vero cells infected with HSV-2 was extracted at 16 hpi. ICP34.5 mRNAs were detected by RT-PCR using exon 1 and intron-specific primers (oST432 and oST398) or exon 1 and exon 2-specific primers (oST432 and oST426). The PCR products were cloned into the pCR4 Topo cloning vector and verified by sequencing before being subcloned into a pFlag expression vector to construct pICP34.5 α , pICP34.5 β and pICP34.5-full as described in Materials and Methods. The relative positions of primers used are labeled in the upper panel. The asterisk (*) indicates a stop codon. (C) HSV-2 ICP34.5 β protein is not efficiently expressed in cell cultures transfected with a full-length ICP34.5 DNA clone (pICP34.5-full) (lane 3). 293 cells were transfected with pICP34.5 α , pICP34.5 β , and pICP34.5-full. Total cell proteins were prepared at 24 h posttransfection. No transfection control cells (NTC) and Vero cells infected with HSV-2 are used as negative and positive controls, respectively.

Fluor 488-conjugated goat anti-rabbit IgG, and Alexa Fluor 555-conjugated goat anti-mouse IgG for 1 h. The chamber slides were washed and mounted before being examined using the Nikon Eclipse Ti fluorescence microscope.

RESULTS

HSV-2 ICP34.5 is an alternatively spliced gene and encodes a novel form of ICP34.5. Western blots with a HSV-2 ICP34.5 specific antibody of proteins from HSV-2-infected cell cultures, in addition to the 38-kDa protein that corresponds to the size of the predicted full-length HSV-2 ICP34.5, showed a 28-kDa protein band. Both the 28- and the 38-kDa proteins were detected as early as 3 h postinfection (hpi), and expression of the 28- and 38-kDa proteins increased dramatically during the late infection (Fig. 1A). The ratio of the 28-kDa bands to 38-kDa bands did not change dramatically as infection progressed. The 28-kDa protein was detected in both early and late infection by an ICP34.5-specific antibody that was raised against ICP34.5-specific short peptides (23), suggesting that the 28-kDa protein contains some of the same peptide sequences and is likely encoded by the HSV-2 ICP34.5 gene. Sequence analysis revealed a premature stop codon in the intron of HSV-2 ICP34.5, which in contrast to HSV-1 ICP34.5, is a spliced gene. We thus hypothesized that the 28-kDa protein is translated from the unspliced ICP34.5 mRNA and named the 38- and 28-kDa proteins ICP34.5 α and ICP34.5 β , respectively. Indeed, in addition to the spliced ICP34.5 mRNA, we detected the unspliced ICP34.5 mRNA in HSV-2-infected cell culture by RT-PCR using primers spanning the splice junction re-

gions and the intron (Fig. 1B), indicating that the ICP34.5 mRNA is not efficiently spliced in infected cell culture. We verified that these PCR products corresponded to spliced and unspliced ICP34.5 mRNAs by gel purifying and Topo cloning the PCR products and sequencing at least 24 clones of each PCR product. These experiments identified no additional splicing site for HSV-2 ICP34.5.

We next generated cytomegalovirus immediate-early (IE) promoter-driven expression constructs containing either the full-length unspliced ICP34.5 (pICP34.5-full) or the spliced cDNA (pICP34.5 α). For the hypothesized unspliced ICP34.5 β product, we also constructed an expression clone containing the entire exon 1 and partial intron sequences with the premature stop codon included (pICP34.5 β). These ICP34.5 expression plasmids were transfected into 293 cells, and their protein products were detected by Western blotting with the ICP34.5-specific antibody. As predicted, both pICP34.5-full and pICP34.5 α expressed the 38-kDa protein, and the pICP34.5 β expressed the 28-kDa protein (Fig. 1C), suggesting that ICP34.5 β is translated from the ICP34.5 unspliced mRNA. The 28-kDa protein was not detected in cells transfected with pICP34.5-full, suggesting that ICP34.5 is efficiently spliced when expressed alone in transfected cells and that the efficient expression of ICP34.5 β may be dependent on viral factors.

HSV-2 ICP34.5 α , but not ICP34.5 β , is capable of reducing the Ser-51 phosphorylation of eIF2 α . Both HSV-1 and HSV-2 ICP34.5 have a conserved C-terminal GADD34-homologous do-

FIG 2 HSV-2 ICP34.5α, but not ICP34.5β, is capable of reducing the Ser-51 phosphorylation of eIF2α. (A) Schematic diagram of HSV-1 and HSV-2 ICP34.5 proteins. An alignment of HSV-1 (top sequence), HSV-2 ICP34.5α (middle sequence), and HSV-2 ICP34.5β (bottom sequence) was performed using Vector NTI (Invitrogen). HSV-2 sequences corresponding to Beclin-1/TBK1 binding and PP1-binding sequences reported for HSV-1 are shown in the middle of the panel. To aid in visual comparisons of the sequences, shared amino acids are enclosed within a box. (B) Unlike ICP34.5α, ICP34.5β does not reduce the Ser-51 phosphorylation of eIF2α induced by R3616, an HSV-1 ICP34.5 deletion mutant. HeLa cells were transfected with pICP34.5β, pICP34.5α, or pFlag vector 24 h before infection with R3616 or its rescuer virus, R3659. Total protein was prepared at 18 hpi. The ratio between the intensities of Ser51-phosphorylated eIF2α and total eIF2α signals was quantified by using ImageQuant TL8.1 software (GE Healthcare).

main, which counteracts PKR-mediated Ser-51 phosphorylation of eIF2 and translation shutdown through interaction with PP1 and phosphorylated eIF2 (p-eIF2) (Fig. 2A). However, ICP34.5β, the novel form of HSV-2 ICP34.5, does not contain the conserved GADD34 domain. Instead, it has an additional 19 aa encoded by the intron (Fig. 2A). The nucleotide sequence for the C-terminal 19 aa and the stop codon is identical between the two viral strains (strains 333 and HG52) for which ICP34.5 sequences are available in GenBank. We used an HSV-1 mutant virus complementation model to test the ability of ICP34.5α and ICP34.5β to reduce Ser-51 phosphorylation. R3616 is a HSV-1 ICP34.5 deletion mutant that has been shown to induce eIF2α phosphorylation in infected cell culture (45, 46). R3659, the rescuer virus for R3616 that contains wild-type HSV-1 ICP34.5 and is thus competent in reducing eIF2α phosphorylation, was also included as a control. We pretransfected HeLa cells with pICP34.5β and pICP34.5α, either alone or in combination, before infection with R3616 or R3659. We found that HSV-2 ICP34.5α, but not ICP34.5β, reduced the Ser-51 phosphorylation of eIF2α induced by R3616

infection (Fig. 2B). When ICP34.5α and ICP34.5β were transfected together, ICP34.5β did not detectably interfere with ICP34.5α's function to reduce Ser-51 phosphorylation of eIF2α (Fig. 2B).

HSV-2 ICP34.5β is predominantly localized in the nucleus, whereas HSV-2 ICP34.5α is mainly localized in the cytoplasm. To better understand the potential function of ICP34.5β, we examined its intracellular localization by immunofluorescence. In Vero cells transfected with various ICP34.5 expression constructs, ICP34.5α is predominantly located in the cytoplasm but also displays a ring-shaped nuclear pattern as shown in subpanels d, e, and f in Fig. 3A. The ring-shaped nuclear pattern resembles the nucleolus, where a fraction of HSV-1 ICP34.5 is also localized (47). ICP34.5β was predominantly detected more diffusely in the nucleus, also with a similar ring-shaped nucleolus-like pattern as shown in subpanels a, b, and c in Fig. 3A, implying that ICP34.5β, the novel form of ICP34.5, may have a biological function different from that of ICP34.5α. ICP34.5 expressed from pICP34.5-full showed intracellular localization identical to that of pICP34.5α, as

FIG 3 ICP34.5β is predominantly localized in the nucleus, whereas ICP34.5α is predominantly located in the cytoplasm. (A) Cellular localization of ICP34.5α and ICP34.5β in transfected cells. Vero cells were transfected with pICP34.5β (a, b and c), pICP34.5α (d, e, and f), or pICP34.5full (g, h, and i). Cells were fixed with 4% paraformaldehyde at 24 h posttransfection prior to incubation with rabbit anti-ICP34.5 antibody, followed by incubation with anti-rabbit IgG-fluorescein isothiocyanate (FITC). Stained cells were examined using a Nikon ECLIPSE Ti fluorescence microscope with a $\times 100$ oil objective lens. (B) Cellular localization of ICP34.5 in HSV-2-infected cells. Vero cells were subjected to HSV-2 strain 333 or mock infection. Cells were fixed with 4% paraformaldehyde at 5 hpi (a, b, and c) or at 16 hpi (d, e, and f) prior to incubation with the rabbit anti-ICP34.5 antibody, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG. Stained cells were observed with a laser scan confocal microscope (Zeiss LSM 510) with a $\times 63$ oil objective lens and a $\times 1.5$ zoom. (C) The nuclear signals representing both pICP34.5α or pICP34.5β colocalize with nucleolin (C23), a nucleolar marker in methanol-fixed cells. U2OS cells transfected with pICP34.5α (a, b, and c) or pICP34.5β (d, e, and f) were fixed with methanol. Cells were then incubated with the rabbit anti-ICP34.5 antibody and monoclonal anti-C23 antibody, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG. Stained cells were observed using the Nikon Eclipse Ti fluorescence microscope with a $\times 100$ oil objective lens.

shown in subpanels g, h, and i in Fig. 3A, consistent with inefficient expression of ICP34.5β in cells transfected with the full-length ICP34.5 genomic sequence. Using laser scanning confocal microscopy with the same anti-ICP34.5 antibody, in HSV-2-infected Vero cells, ICP34.5 proteins (a mixture of ICP34.5β and ICP34.5α) were distributed in both the nucleus and the cytoplasm throughout early and late infection (Fig. 3B). A cytoplasmic pattern, with nuclear densities (also consistent with nucleolar lo-

calization) were observed for ICP34.5 at the early time point (5 hpi). By 16 hpi, the cells were pyknotic and the cytoplasm was considerably smaller than at 5 h. At 16 hpi, ICP34.5 was also detected in both cytoplasm and nucleus. This pattern is different from that reported for HSV-1 ICP34.5, which is predominantly localized in the nucleus early in infection, but shifts to the cytoplasm during late infection (41, 47, 48). The stronger nuclear signal for ICP34.5 in HSV-2-infected Vero cells is consistent in loca-

FIG 4 HSV-2 ICP34.5 is efficiently spliced in transfected cells; however, ICP34.5 splicing is dramatically inhibited by HSV-2 infection. Vero cells were transfected with pICP34.5-full prior to HSV-2 or mock infection at an MOI of 2. cDNAs were prepared using total RNA at 16 h posttransfection. RT-PCR was performed using specific primers indicated in the figure. Primer sets specific to expression vector or to HSV-2 were used to detect ICP34.5 transcripts from pICP34.5-full or virus, respectively. RT-PCR using the same set of cDNAs, and primer sets specific to GAPDH, Bcl-XL/S, and β -actin were used as loading and endogenous splicing efficiency controls.

tion with that of ICP34.5 β in transfected cells, suggesting that ICP34.5 β is expressed by virus in infected cells and that the regulation of intracellular localization of HSV-2 ICP34.5 during viral infection may be different from that of HSV-1 ICP34.5.

A fraction of HSV-1 ICP34.5 is also detected in the nucleolus (47). To verify that HSV-2 ICP34.5 is partly localized in the nucleolus, we costained U2OS cells transfected with ICP34.5 β and ICP34.5 α expression constructs with both the anti-ICP34.5 antibody and an antibody against nucleolin (C23). In cells fixed with formalin or paraformaldehyde, C23 is reported to be detected in the nucleus, with a diffuse pattern. However, immunofluorescence staining of methanol-fixed cells using anti-C23 identifies the nucleolus (although the typically ring-shaped nucleolar structure collapses into a denser structure), indicating that nucleolin is a specific nucleolar marker when using methanol-fixed cells (47). We found that a fraction of both ICP34.5 α and ICP34.5 β colocalized with C23 when cells were fixed with methanol, confirming that a fraction of HSV-2 ICP34.5 is localized in nucleoli, which are relatively more abundant in U2OS cells (Fig. 3C).

Efficient expression of HSV-2 ICP34.5 β is dependent on ICP27. Based on the observation that ICP34.5 β is efficiently expressed in virus-infected cells but not in cells transfected with pICP34.5-full, which contains the full-length ICP34.5 genomic sequence, we hypothesized that viral infection may be required for ICP34.5 β expression. To test this hypothesis, we transfected Vero cells with pICP34.5-full and examined ICP34.5 splicing in the presence or absence of HSV-2 infection using RT-PCR. To distinguish the ICP34.5 mRNAs expressed by the transfected pICP34.5-full from those expressed by virus, we designed two sets of primers

for ICP34.5, with one set specifically detecting ICP34.5 mRNAs expressed from pICP34.5-full and the other set for ICP34.5 mRNAs expressed from HSV-2. We also used specific primers flanking constitutively spliced or alternatively spliced introns of cellular spliced genes including β -actin, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and Bcl-XL/S as controls. RT-PCR results confirmed that ICP34.5 expressed from pICP34.5-full is spliced efficiently in cells without viral infection (Fig. 4), which explains why ICP34.5 β protein is not efficiently expressed under this condition (Fig. 1). However, ICP34.5 mRNA expressed by pICP34.5-full is not efficiently spliced in HSV-2-infected cells (Fig. 4), suggesting that ICP34.5 splicing is dramatically inhibited by viral infection. Splicing of the ICP34.5 mRNA expressed by the transfected pICP34.5-full was inhibited to a larger extent than that of the ICP34.5 expressed from the virus (Fig. 4). Similar results were also observed when replacing HSV-2 with HSV-1 infection in the pICP34.5-full transfected cells (data not shown). In contrast, the splicing of several constitutively or alternatively spliced cellular genes including β -actin, GAPDH, and Bcl-XL/S was not appreciably modified by viral infection. These results suggested that the inhibition of the ICP34.5 splicing by HSV-2 is specific to ICP34.5 and might not be a result of a general inhibition of splicing by viral infection (Fig. 4).

Since ICP27 was reported to modify general splicing and alternative splicing (33), we tested for a potential role of ICP27 in the inhibition of HSV-2 ICP34.5 pre-mRNA splicing. In a transient transfection experiment, we found that coexpression of HSV-2 ICP27 and ICP34.5 resulted in a dramatic increase in the levels of unspliced ICP34.5 mRNA and a reduction in that of the spliced

FIG 5 Splicing of ICP34.5 is inhibited by HSV-2 ICP27, a multifunctional immediate-early protein. (A) HSV-2 ICP27 increases the unspliced form of ICP34.5 mRNAs. 293 cells were cotransfected with pICP34.5-full and either pICP27 or pFlag vector. cDNAs were prepared using total RNAs at 24 h posttransfection. RT-PCR was performed using the same set of cDNAs and the specific primers indicated in the figures. (B) ICP27 promotes ICP34.5 β protein expression. 293 cells were cotransfected with pICP34.5-full and either pICP27 or pFlag vector. Total protein was prepared at 18 h posttransfection. The same membrane was blotted with an anti- β -tubulin antibody and a monoclonal anti-ICP27 antibody after stripping. (C) Inhibition of ICP34.5 splicing by ICP27 is much more efficient than that of other reporters, including KSHV K8 and HPV16 E6E7 genes. 293 cells were cotransfected with pICP34.5-full, pST1 (KSHV K8 gene reporter), or pWX1 (HPV16 E6E7 gene reporter) and either pICP27 or pFlag vector. cDNAs were prepared using total RNAs at 24 h posttransfection. RT-PCR was performed using the same set of cDNAs and specific primers indicated in the figure.

form, indicative of splicing inhibition (Fig. 5A). In contrast, the splicing patterns of cellular genes, such as alternatively spliced Bcl-XL/S and constitutively spliced GAPDH and β -actin, were not altered by ICP27 (Fig. 5A). These results indicate that ICP27 inhibits ICP34.5 splicing in a gene-specific manner. Furthermore, at the protein level, cotransfection of ICP27 and ICP34.5 reduced the expression of ICP34.5 α but increased the expression of ICP34.5 β (Fig. 5B), which is consistent with their mRNA levels shown by the RT-PCR results (Fig. 5A).

To further confirm that inhibition of ICP34.5 alternative splicing by ICP27 is specific, we cotransfected 293 cells with pICP34.5-full and two known alternatively spliced gene reporters, pST1 (44), a KSHV K8 α and β gene reporter, and pWX1 (43), a HPV16 E6/E7 gene reporter, with or without pICP27. The RT-PCR results confirmed that ICP34.5 splicing was inhibited by ICP27 (Fig. 5C). However, alternative splicing of neither KSHV K8 nor HPV16 E6/E7 was affected by ICP27 as examined by RT-PCR using specific primers and the same cDNAs (Fig. 5C). The cellular GAPDH splicing pattern was also not affected, consistent with findings shown in Fig. 5A and further confirming that ICP27 specifically modifies ICP34.5 alternative splicing, promoting ICP34.5 β expression and inhibiting ICP34.5 α expression.

The C-terminal domain of ICP27 is required for its specific inhibition of ICP34.5 splicing, as well as for promotion of ICP34.5 β expression. ICP27 is a multifunctional protein, and many of its functions have been mapped to different regions of the protein (33). To further dissect which region of ICP27 mediates its inhibitory effect on ICP34.5 splicing, we tested the impact of various HSV-2 ICP27 mutants (including d1-2, Δ RGG, Δ RR2, and M15) on the ICP34.5 splicing pattern by RT-PCR in a cotransfection experiment using pICP34.5. A diagram of these ICP27 mutants is shown in Fig. 6A. ICP27 mutants either with deletion of the majority of the nuclear export signal (d1-2) or deletions of the RNA-binding domains (Δ RGG and Δ RR2) retained the ability to inhibit ICP34.5 splicing (Fig. 6B). However, a 2-aa mutation at the C terminus of ICP27 (M15) abolished its inhibitory effect on ICP34.5 splicing as detected by a reduction in the level of the unspliced transcript, demonstrating the importance of the C-terminal KH3 domain of ICP27 in inhibition of ICP34.5 splicing. By Western blotting, d1-2, Δ RGG, and Δ RR2 mutants increased expression of ICP34.5 β (Fig. 6B). However, the level of ICP34.5 β protein was significantly lower in cells transfected with the M15 mutant than with the other mutants and the wild-type ICP27 transfected cells. These results were consistent with the levels of

FIG 6 The C terminus of HSV-2 ICP27 is required for inhibition of HSV-2 ICP34.5 alternative splicing. (A) Schematic diagram of ICP27 mutants used in panel B. d1-2 contains a partial deletion of NES (nuclear exporting signal) sequences and the ECS (exporting control signal) at aa 34 to 35. ΔRR2 has additional deletions of the arginine-rich sequence downstream of the RGG box motif. M15 contains two amino acid mutations (Pro466Leu and Gly467Glu) at the KH3 domain. Abbreviations: RGG box, RNA binding motif that contains repeats of Arg-Gly-Gly; KH domain, human heterogeneous nuclear ribonucleoprotein (hnRNP) K homolog domain. (B) M15 with a 2-aa mutation in the ICP27 C terminus fails to inhibit ICP34.5 splicing and promote ICP34.5β expression. 293 cells were cotransfected with pICP34.5-full and either pFlag vector, pICP27 (WT), d1-2, ΔRGG, ΔRR2, or M15. Total RNA and protein were prepared 24 h posttransfection. RT-PCR results using the primers oST432 and oST426 indicate that ICP27 mutants, including d1-2, ΔRGG, and ΔRR2, but not M15, could inhibit ICP34.5 splicing. The Western blot result (the middle panel) further confirms that M15 is the least efficient in promoting ICP34.5β expression and inhibiting ICP34.5α expression. The same membrane was blotted with the monoclonal anti-ICP27 antibody after stripping.

the unspliced ICP34.5 mRNA shown by RT-PCR (Fig. 6B). The expression levels of the ICP27 mutants were comparable, as indicated by Western blotting using a monoclonal anti-ICP27 antibody (H113). A protein smaller than wild-type or mutant ICP27 was also detected by the ICP27 antibody. A similar band was also detected in HSV-2-infected cell cultures (data not shown). Only one band was detected by a flag-specific antibody when flag-tagged ICP27 was expressed (data not shown), suggesting the smaller band is likely a degradation product of ICP27. Therefore, both RT-PCR and Western blot results demonstrated that the C terminus of ICP27 is required for its specific inhibition of ICP34.5 splicing as well as promotion of ICP34.5β expression.

DISCUSSION

The mechanism by which ICP34.5 promotes viral neurovirulence is not clear. In the present study, in addition to the full-length ICP34.5, we identified a novel form of HSV-2 ICP34.5, denoted ICP34.5β. HSV-2 ICP34.5β is translated from the unspliced ICP34.5 mRNA, is detectable as early as 3 hpi, and accumulates dramatically during late viral infection. Efficient expression of ICP34.5β is dependent on expression of ICP27, a multifunctional immediate-earlier gene. ICP27 specifically inhibits ICP34.5 splicing and promotes ICP34.5β expression.

The C-terminal domain of ICP27 is critical for its inhibition on the ICP34.5 alternative splicing.

HSV-2 ICP34.5α and ICP34.5β are distributed among the cytoplasm and nucleus. However, ICP34.5β is predominantly located in the nucleus (mostly in nucleoli), while ICP34.5α is predominantly located in cytoplasm. HSV-1 ICP34.5 has been shown to contain a nuclear localization signal (NLS; aa 188 to 206) within the GADD34 domain, a nucleolar localization signal (aa 1 to 16), and a nuclear exporting signal (NES; aa 128 to 137) (41, 47). The nucleolar localization signal (aa 1 to 16) also functions as an NLS. These localization signal sequences also mapped to HSV-2 ICP34.5α by sequence homology analysis. However, HSV-2 ICP34.5β does not contain the GADD34 domain and thus lacks the NLS embedded in this region. ICP34.5β does contain the nucleolar localization signal (aa 1 to 16) and the NES. The fact that ICP34.5α also showed a strong cytoplasmic signal suggests that, in addition to the NES, sequences in the C-terminal domain of ICP34.5α (likely in addition to the NLS, which would provide for nuclear localization) are important for its cytoplasmic localization. The nuclear localization of ICP34.5β suggests that it may contain an additional nuclear localization signal or lack additional nuclear export element. The presence of multiple localization signals in each protein suggests that they may both be capable of

shuttling between cytoplasm and nucleus. HSV-2 ICP34.5 lacks the central proline-alanine-threonine (PAT) repeats, which are required for HSV-1 neuroinvasion (42). The number of PAT repeats also likely contributes to the subcellular localization of HSV-1 ICP34.5 (41) and efficient viral egress (40). It remains to be determined how HSV-2 ICP34.5 α and ICP34.5 β retain their neuroinvasion function without the PAT repeats.

The efficient expression of ICP34.5 β is dependent on the specific inhibition of ICP34.5 splicing by ICP27 or viral infection. It has been reported that HSV-1 ICP27, a conserved homolog of HSV-2 ICP27, can inhibit global gene splicing through inhibition of spliceosomal assembly (33). However, we observed that ICP27 inhibits ICP34.5 splicing much more efficiently than it inhibits other constitutive or alternative splicing of viral or cellular genes, suggesting that the specific inhibition of ICP34.5 splicing is not due to global splicing inhibition. Furthermore, we demonstrated that the C-terminal domain of ICP27 is critical for the inhibition of ICP34.5 splicing as mutation of two amino acids in the C-terminal KH3 domain (M15) abolishes the ICP27 effect on ICP34.5 splicing and consequently the ICP34.5 β protein expression level (Fig. 6B). Recently, HSV-1 ICP27 was reported to inhibit the splicing of glycoprotein C (gC) and thus to reduce the expression of soluble gC, the product of the alternatively spliced transcript (35, 49). In transfected cells, expression of both full-length and the truncated soluble gC requires the presence of ICP27 to counteract the effect of a *cis*-acting inhibitory sequence in the gC gene (49). However, the expression of ICP34.5 α in transfected cells is independent of ICP27, suggesting a different mechanism. HSV-2 ICP27 was also reported to modify promyelocytic leukemia (PML) isoforms by altering pre-mRNA splicing (34). Nojima et al. proposed that the weak 3' splice site of the PML intron 7a is a specific target for splicing inhibition by ICP27. However, we did not identify similar suboptimal splicing sites in ICP34.5, a finding suggestive of alternative inhibition mechanisms. Further study of the mechanism by which ICP27 specifically inhibits ICP34.5 splicing may help unveil more viral and cellular alternative splicing targets for ICP27.

Several key functions of HSV-1 ICP34.5, including inhibition of autophagy through binding to Beclin-1 and inhibition of transforming growth factor β expression through binding to TBK1, have been mapped to its N-terminal region. The Beclin-1 and TBK1 binding sequences in HSV-1 ICP34.5 are partially overlapping (Fig. 2A). It is not known whether the relatively nonhomologous N-terminal domain of HSV-2 ICP34.5 has similar functions, but it seems unlikely that this region would account for differences in function between ICP34.5 α and ICP34.5 β because it is present in both. It was reported that compared to the wild-type virus, an ICP34.5 intron deletion mutant has similar replication efficiency *in vitro* and similar neurovirulence in an intracerebral murine model (39). Thus, it seems likely that any effect of inhibiting ICP34.5 splicing to express ICP34.5 β was not readily detectable in these assays. One possibility is that inhibition of ICP34.5 splicing reduces ICP34.5 α expression and thus reduces neurovirulence, implying that an intron-deletion mutant might be predicted to be more neurovirulent than wild-type virus. These experiments were not designed to detect such outcomes, considering the high baseline neurovirulence of HSV-2 and the absence of a rescuant virus. It is also possible that upregulated ICP34.5 α in ICP34.5 intron deletion mutant-infected cells could compensate for the loss of ICP34.5 β function in these models. Further exper-

iments are needed in order to examine the role of ICP34.5 splicing in the virus life cycle.

The best-characterized functions for ICP34.5 to date are cytoplasm-related functions. A potential nuclear function of ICP34.5 has been hypothesized. HSV-1 ICP34.5 was reported to interact with PCNA-1, a nucleolar protein in the late S phase of the cell cycle, through binding of its C-terminal conserved sequences and possibly also the N-terminal region (48, 50). Given the fact that ICP34.5 β lacks the C-terminal conserved domain, it seems unlikely that ICP34.5 β interacts with PCNA-1, although both show nucleolar localization. Therefore, further study of the nuclear function of ICP34.5 β is needed to improve understanding of the nuclear function of ICP34.5.

Although it is still not known whether ICP27 also influences ICP34.5 expression at the transcriptional level, it is clear that ICP27 posttranscriptionally regulates HSV-2 ICP34.5 α and ICP34.5 β expression levels and their ratio. As expression of the unspliced ICP34.5 mRNA encoding ICP34.5 β increases, spliced ICP34.5 mRNA encoding ICP34.5 α is reduced by ICP27 (Fig. 5 and 6). Similarly, ICP34.5 α protein expression is inhibited while ICP34.5 β protein expression is promoted by ICP27, suggesting that the levels of different ICP34.5 isoforms are regulated by ICP27 (Fig. 5 and 6). ICP34.5 is one of a few spliced genes that are conserved in the HSV-2 strains (333 and HG52) for which ICP34.5 sequence is available, suggesting the evolutionary importance of this splicing mechanism. In contrast, an average human gene contains between 8 and 9 introns, and there are approximately 190,159 introns in 21,746 protein-coding genes in the human genome (51). The fact that ICP27 inhibits ICP34.5 splicing much more efficiently and specifically than it does other cellular spliced genes suggests that such regulation of ICP34.5 by ICP27 likely plays a role in viral pathogenesis. We speculate that expression of ICP34.5 β , which contains the less conserved N-terminal sequences, offers an additional important nuclear function.

Since HSV-1 ICP34.5 does not encode an intron, it cannot be regulated by ICP27-mediated inhibition of splicing, suggesting that ICP27 plays somewhat different roles in HSV-1 and HSV-2. HSV-1 ICP27 was previously shown to inhibit HSV-1 ICP0 splicing and promote expression of a truncated protein through intron-retention retention (52–54). Since HSV-1 and HSV-2 ICP27 have an overall homology of 83% and the HSV-1 and HSV-2 ICP27 C-terminal domain that in HSV-2 is important for specific inhibition of ICP34.5 splicing is even more highly conserved with ~94% homology, it will be interesting to determine whether HSV-2 ICP27 also plays a role in HSV-2 ICP0 splicing. We are currently investigating whether differences in how HSV-1 and HSV-2 ICP27 interacts with ICP0, ICP34.5, and ICP4 could potentially account for differences in HSV-1 and HSV-2 phenotypes.

We previously reported that HSV-2 ICP34.5 expression is likely regulated by two acutely and latently expressed viral miRNAs produced from the LAT promoter (15, 16). In addition, ICP34.5 expression may also be regulated by ICP4-controlled L/ST expression (10–12), possibly also through miRNAs (16). These findings strongly suggest that as an important neurovirulence factor, expression of ICP34.5 is under tight control by multiple mechanisms that contribute to the most efficient viral life cycle. Further work is needed to better understand the mechanism by which ICP27 regulates HSV-2 ICP34.5 splicing and the potential contribution of ICP34.5 β to the neurovirulence and recurrence phenotypes of HSV-2.

ACKNOWLEDGMENTS

We thank Shasta McClenahan, Keith Peden, and Haruhiko Murata for critical readings of the manuscript. We also thank Bernard Roizman for providing the ICP34.5 deletion mutant (R3616) and its rescuant (R3659). We also thank Masatoshi Hagiwara and Takayuki Nojima for providing the HSV-2 ICP27 mutant plasmids and Zhiming Zheng for providing KSHV K8 and HPV E6E7 expression plasmids.

This study was supported by the intramural research programs of the Center for Biologics Evaluation and Research.

REFERENCES

- Chou J, Roizman B. 1994. Herpes simplex virus 1 gamma (1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proc. Natl. Acad. Sci. U. S. A.* 91:5247–5251.
- Chou J, Kern ER, Whitley RJ, Roizman B. 1990. Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. *Science* 250:1262–1266.
- Chou J, Roizman B. 1992. The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl. Acad. Sci. U. S. A.* 89:3266–3270.
- Whitley RJ, Kern ER, Chatterjee S, Chou J, Roizman B. 1993. Replication, establishment of latency, and induced reactivation of herpes simplex virus gamma 1 34.5 deletion mutants in rodent models. *J. Clin. Invest.* 91:2837–2843.
- Markert JM, Medlock MD, Rabkin SD, Gillespie GY, Todo T, Hunter WD, Palmer CA, Feigenbaum F, Tornatore C, Tufaro F, Martuza RL. 2000. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther.* 7:867–874.
- Ramplung R, Cruickshank G, Papanastassiou V, Nicoll J, Hadley D, Brennan D, Petty R, MacLean A, Harland J, McKie E, Mabbs R, Brown M. 2000. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther.* 7:859–866.
- Spivack JG, Fareed MU, Valyi-Nagy T, Nash TC, O'Keefe JS, Gesser RM, McKie EA, MacLean AR, Fraser NW, Brown SM. 1995. Replication, establishment of latent infection, expression of the latency-associated transcripts and explant reactivation of herpes simplex virus type 1 gamma 34.5 mutants in a mouse eye model. *J. Gen. Virol.* 76(Pt 2):321–332.
- Chou J, Roizman B. 1986. The terminal a sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. *J. Virol.* 57:629–637.
- Holman HA, MacLean AR. 2008. Neurovirulent factor ICP34.5 uniquely expressed in the herpes simplex virus type 1 Delta gamma 1 34.5 mutant 1716. *J. Neurovirol.* 14:28–40.
- Lee LY, Schaffer PA. 1998. A virus with a mutation in the ICP4-binding site in the L/ST promoter of herpes simplex virus type 1, but not a virus with a mutation in open reading frame P, exhibits cell-type-specific expression of gamma(1)34.5 transcripts and latency-associated transcripts. *J. Virol.* 72:4250–4264.
- Randall G, Lagunoff M, Roizman B. 1997. The product of ORF O located within the domain of herpes simplex virus 1 genome transcribed during latent infection binds to and inhibits in vitro binding of infected cell protein 4 to its cognate DNA site. *Proc. Natl. Acad. Sci. U. S. A.* 94:10379–10384.
- Randall G, Roizman B. 1997. Transcription of the derepressed open reading frame P of herpes simplex virus 1 precludes the expression of the antisense gamma(1)34.5 gene and may account for the attenuation of the mutant virus. *J. Virol.* 71:7750–7757.
- Jurak I, Kramer MF, Mellor JC, van Lint AL, Roth FP, Knipe DM, Coen DM. 2010. Numerous conserved and divergent microRNAs expressed by herpes simplex viruses 1 and 2. *J. Virol.* 84:4659–4672.
- Tang S, Bertke AS, Patel A, Margolis TP, Krause PR. Herpes simplex virus 2 microRNA miR-H6 is a novel latency-associated transcript-associated microRNA, but reduction of its expression does not influence the establishment of viral latency or the recurrence phenotype. *J. Virol.* 85:4501–4509.
- Tang S, Bertke AS, Patel A, Wang K, Cohen JI, Krause PR. 2008. An acutely and latently expressed herpes simplex virus 2 viral microRNA inhibits expression of ICP34.5, a viral neurovirulence factor. *Proc. Natl. Acad. Sci. U. S. A.* 105:10931–10936.
- Tang S, Patel A, Krause PR. 2009. Novel less-abundant viral microRNAs encoded by herpes simplex virus 2 latency-associated transcript and their roles in regulating ICP34.5 and ICP0 mRNAs. *J. Virol.* 83:1433–1442.
- Umbach JL, Kramer MF, Jurak I, Karnowski HW, Coen DM, Cullen BR. 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* 454:780–783.
- Umbach JL, Wang K, Tang S, Krause PR, Mont EK, Cohen JI, Cullen BR. Identification of viral microRNAs expressed in human sacral ganglia latently infected with herpes simplex virus 2. *J. Virol.* 84:1189–1192.
- He B, Chou J, Liebermann DA, Hoffman B, Roizman B. 1996. The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the gamma(1)34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. *J. Virol.* 70:84–90.
- He B, Gross M, Roizman B. 1997. The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* 94:843–848.
- Brown SM, Harland J, MacLean AR, Podlech J, Clements JB. 1994. Cell type and cell state determine differential in vitro growth of non-neurovirulent ICP34.5-negative herpes simplex virus types 1 and 2. *J. Gen. Virol.* 75(Pt 9):2367–2377.
- Orvedahl A, Alexander D, Tallozy Z, Sun Q, Wei Y, Zhang W, Burns D, Leib DA, Levine B. 2007. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* 1:23–35.
- Tallozy Z, Virgin HW, IV, Levine B. 2006. PKR-dependent autophagic degradation of herpes simplex virus type 1. *Autophagy* 2:24–29.
- Ma Y, Jin H, Valyi-Nagy T, Cao Y, Yan Z, He B. Inhibition of TANK binding kinase 1 by herpes simplex virus 1 facilitates productive infection. *J. Virol.* 86:2188–2196.
- Verpooten D, Ma Y, Hou S, Yan Z, He B. 2009. Control of TANK-binding kinase 1-mediated signaling by the gamma(1)34.5 protein of herpes simplex virus 1. *J. Biol. Chem.* 284:1097–1105.
- Brown SM, MacLean AR, Aitken JD, Harland J. 1994. ICP34.5 influences herpes simplex virus type 1 maturation and egress from infected cells in vitro. *J. Gen. Virol.* 75(Pt 12):3679–3686.
- Jing X, Cervený M, Yang K, He B. 2004. Replication of herpes simplex virus 1 depends on the gamma 134.5 functions that facilitate virus response to interferon and egress in the different stages of productive infection. *J. Virol.* 78:7653–7666.
- Rice SA, Knipe DM. 1988. Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. *J. Virol.* 62:3814–3823.
- Sacks WR, Greene CC, Aschman DP, Schaffer PA. 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *J. Virol.* 55:796–805.
- Sandri-Goldin RM, Hibbard MK. 1996. The herpes simplex virus type 1 regulatory protein ICP27 coimmunoprecipitates with anti-Sm antiserum, and the C terminus appears to be required for this interaction. *J. Virol.* 70:108–118.
- Sciabica KS, Dai QJ, Sandri-Goldin RM. 2003. ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation. *EMBO J.* 22:1608–1619.
- Bryant HE, Wadd SE, Lamond AI, Silverstein SJ, Clements JB. 2001. Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. *J. Virol.* 75:4376–4385.
- Sandri-Goldin RM. 2011. The many roles of the highly interactive HSV protein ICP27, a key regulator of infection. *Future Microbiol.* 6:1261–1277.
- Nojima T, Oshiro-Ideue T, Nakanoya H, Kawamura H, Morimoto T, Kawaguchi Y, Kataoka N, Hagiwara M. 2009. Herpesvirus protein ICP27 switches PML isoform by altering mRNA splicing. *Nucleic Acids Res.* 37:6515–6527.
- Sedlackova L, Perkins KD, Lengyel J, Strain AK, van Santen VL, Rice SA. 2008. Herpes simplex virus type 1 ICP27 regulates expression of a variant, secreted form of glycoprotein C by an intron retention mechanism. *J. Virol.* 82:7443–7455.
- McKendall RR. 1980. Comparative neurovirulence and latency of HSV1 and HSV2 following footpad inoculation in mice. *J. Med. Virol.* 5:25–32.

37. Plummer G, Waner JL, Bowling CP. 1968. Comparative studies of type 1 and type 2 and 'herpes simplex' viruses. *Br. J. Exp. Pathol.* **49**:202–208.
38. Scriba M. 1981. Persistence of herpes simplex virus (HSV) infection in ganglia and peripheral tissues of guinea pigs. *Med. Microbiol. Immunol.* **169**:91–96.
39. Ravi V, Kennedy PG, MacLean AR. 1998. Functional analysis of the herpes simplex virus type 2 strain HG52 RL1 gene: the intron plays no role in virulence. *J. Gen. Virol.* **79**(Pt 7):1613–1617.
40. Jing X, He B. 2005. Characterization of the triplet repeats in the central domain of the γ 134.5 protein of herpes simplex virus 1. *J. Gen. Virol.* **86**:2411–2419.
41. Mao H, Rosenthal KS. 2002. An N-terminal arginine-rich cluster and a proline-alanine-threonine repeat region determine the cellular localization of the herpes simplex virus type 1 ICP34.5 protein and its ligand, protein phosphatase 1. *J. Biol. Chem.* **277**:11423–11431.
42. Mao H, Rosenthal KS. 2003. Strain-dependent structural variants of herpes simplex virus type 1 ICP34.5 determine viral plaque size, efficiency of glycoprotein processing, and viral release and neuroinvasive disease potential. *J. Virol.* **77**:3409–3417.
43. Tang S, Tao M, McCoy JP, Jr, Zheng ZM. 2006. The E7 oncoprotein is translated from spliced E6*1 transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell lines via translation reinitiation. *J. Virol.* **80**:4249–4263.
44. Tang S, Zheng ZM. 2002. Kaposi's sarcoma-associated herpesvirus K8 exon 3 contains three 5'-splice sites and harbors a K8.1 transcription start site. *J. Biol. Chem.* **277**:14547–14556.
45. Cheng G, Yang K, He B. 2003. Dephosphorylation of eIF-2 α mediated by the gamma(1)34.5 protein of herpes simplex virus type 1 is required for viral response to interferon but is not sufficient for efficient viral replication. *J. Virol.* **77**:10154–10161.
46. Chou J, Chen JJ, Gross M, Roizman B. 1995. Association of an M₁ 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 α and premature shutoff of protein synthesis after infection with gamma 134.5 mutants of herpes simplex virus 1. *Proc. Natl. Acad. Sci. U. S. A.* **92**:10516–10520.
47. Cheng G, Brett ME, He B. 2002. Signals that dictate nuclear, nucleolar, and cytoplasmic shuttling of the gamma(1)34.5 protein of herpes simplex virus type 1. *J. Virol.* **76**:9434–9445.
48. Harland J, Dunn P, Cameron E, Conner J, Brown SM. 2003. The herpes simplex virus (HSV) protein ICP34.5 is a virion component that forms a DNA-binding complex with proliferating cell nuclear antigen and HSV replication proteins. *J. Neurovirol.* **9**:477–488.
49. Sedlackova L, Perkins KD, Meyer J, Strain AK, Goldman O, Rice SA. 2010. Identification of an ICP27-responsive element in the coding region of a herpes simplex virus type 1 late gene. *J. Virol.* **84**:2707–2718.
50. Brown SM, MacLean AR, McKie EA, Harland J. 1997. The herpes simplex virus virulence factor ICP34.5 and the cellular protein MyD116 complex with proliferating cell nuclear antigen through the 63-amino-acid domain conserved in ICP34.5, MyD116, and GADD34. *J. Virol.* **71**:9442–9449.
51. Fedorova I, Fedorov A. 2005. Puzzles of the human genome: why do we need our introns? *Curr. Genomics* **6**:589–595.
52. Ellison KS, Rice SA, Verity R, Smiley JR. 2000. Processing of alpha-globin and ICP0 mRNA in cells infected with herpes simplex virus type 1 ICP27 mutants. *J. Virol.* **74**:7307–7319.
53. Spatz SJ, Nordby EC, Weber PC. 1996. Mutational analysis of ICP0R, a transrepressor protein created by alternative splicing of the ICP0 gene of herpes simplex virus type 1. *J. Virol.* **70**:7360–7370.
54. Weber PC, Kenny JJ, Wigdahl B. 1992. Antiviral properties of a dominant-negative mutant of the herpes simplex virus type 1 regulatory protein ICP0. *J. Gen. Virol.* **73**(Pt 11):2955–2961.