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Dephosphorylation of eIF2α Mediated by the $\gamma_1$34.5 Protein of Herpes Simplex Virus 1 Facilitates Viral Neuroinvasion▽

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The $\gamma_1$34.5 protein, a virulence factor of herpes simplex viruses, redirects protein phosphatase 1 to dephosphorylate the α subunit of translation initiation factor 2 (eIF2α). Additionally, it inhibits the induction of antiviral genes by TANK-binding kinase 1. Nevertheless, its precise role in vivo remains to be established. Here we show that eIF2α dephosphorylation by $\gamma_1$34.5 is crucial for viral neuroinvasion. $V^{193}E$ and $F^{195}L$ substitutions in $\gamma_1$34.5 abrogate viral replication in the eye and spread to the trigeminal ganglia and brain. Intriguingly, inhibition of antiviral gene induction by $\gamma_1$34.5 is not sufficient to exhibit viral virulence.

Herpes simplex viruses (HSV) are human pathogens responsible for a variety of diseases, including genital herpes, keratitis, and encephalitis (28). It has been established that $\gamma_1$34.5 is essential for viral virulence (7, 19). HSV $\gamma_1$34.5 is a multifunctional protein involved in different processes of HSV infection, such as dephosphorylation of the α subunit of translation initiation factor 2 (eIF2α), major histocompatibility complex class II expression, autophagy, and virus egress (2, 14, 22, 25). We have noted that $\gamma_1$34.5 suppresses the maturation of dendritic cells and the induction of antiviral genes, where it targets TANK-binding kinase 1 (TBK1), a key component of Toll-like receptor (TLR)-related pathways (17, 26). Notably, in HSV-infected cells, $\gamma_1$34.5 also prevents translation arrest mediated by the double-stranded RNA-dependent protein kinase (PKR) (6, 8). This is accomplished by $\gamma_1$34.5 recruiting protein phosphatase 1 (PP1) to dephosphorylate eIF2α (13, 14). In this context, it has been demonstrated that the $\gamma_1$34.5 null mutant is virulent in $PKR^{-/-}$ mice but not in wild-type mice (7, 18). PKR is a component integrating innate signaling pathways leading to translation arrest and the expression of proinflammatory cytokines (11, 12, 16, 21). In addition to eIF2α phosphorylation, PKR has a broad range of regulatory functions, which include the activation of NF-κB and interferon regulatory factor 3 (IRF3) in response to signals of TLRs or cytosolic RNA sensors (11, 12, 16, 20, 21, 30). Accordingly, deletion of PKR not only impairs eIF2α phosphorylation but also has a compounding effect on the aforementioned events. Further, removal of $\gamma_1$34.5 from HSV may disrupt more than one viral function. This study was undertaken to further define the role of $\gamma_1$34.5 in HSV neuroinvasion.

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Early studies revealed that $\gamma_1$34.5 is a corneal virulence factor (1, 27). Since $\gamma_1$34.5 is thought to function via the PP1 binding and effector domains (3), we asked whether the PP1 binding domain had a role in HSV infection in vivo. We focused on a recombinant virus, H9813, which bears $V^{193}E$ and $F^{195}L$ substitutions in $\gamma_1$34.5 (4). Such mutations disrupt the interaction of $\gamma_1$34.5 and PP1 (29). As controls, we included wild-type HSV-1(F) and R3616, which has deletion of the entire $\gamma_1$34.5 gene (7). Mice were infected with 4×10^5 PFU of HSV-1(F), R3616, or H9813 through bilateral corneal scarification. At 5 days postinfection, viral yields in different tissues were determined. Figure 1A shows that HSV-1(F) replicated efficiently in the eye, with a titer of 2.6×10^3 PFU. Additionally, the virus was able to travel to the trigeminal ganglia and brain stem, reaching titers of 6.7×10^2 PFU and 1.9×10^3 PFU, respectively. In contrast, R3616 failed to replicate in the eye, with a titer of 1.5 PFU. Infectious viruses were not detectable in the trigeminal ganglia and brain stem. A similar phenotype was seen for H9813. The results of immunohistochemical staining of tissue sections correlated with these phenotypes (Fig. 1B). All mice infected with HSV-1(F) had positive staining in the three tissue types tested. For the R3616 and H9813 viruses, positive staining was observed only in the eye, for two and one out of six mice, respectively. Trigeminal ganglia and brain stem tissues were negative for both viruses.

We further examined the kinetics of in vivo viral replication on days one, three, and seven. Data in Fig. 2A show that HSV-1(F) replicated efficiently in the eye on day one, reaching a titer of 2.4×10^4 PFU. As infection continued, HSV-1(F) maintained viral yields at 1.1×10^3 PFU and 3.2×10^2 PFU in the eye on days three and seven. The gradual reduction of viral replication probably resulted from the activation of host responses as infection continued. In this period, neither R3616 nor H9813 replicated at an appreciable level from the onset, reaching a meager titer of 1.8 PFU over the course of infection. In the trigeminal ganglia (Fig. 2B), HSV-1(F) appeared on day one with a titer of 5.6 PFU and replicated to a peak titer of 8.3×10^2 PFU on day three, indicating that wild-type virus spread efficiently to the trigeminal ganglia. By day seven, the

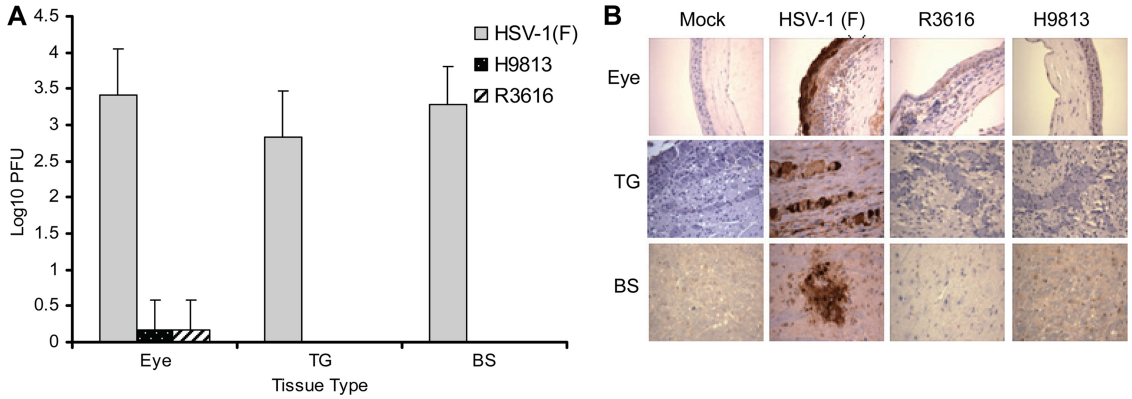

FIG. 1. (A) Viral replication in the eye, trigeminal ganglia, and brain. Groups of 6-week-old female BALB/c mice were mock infected or infected with HSV-1(F), R3616, or H9813 at 4×10^5 PFU through corneal scarification. At 5 days postinfection, eye, trigeminal ganglia (TG), and brain stem (BS) tissues were collected to determine virus yields. Data are expressed as means ± standard deviations for six mice for each group. (B) Immunohistochemistry staining of mouse tissues. The sections from eye, trigeminal ganglia, and brain stem tissues described above were reacted with anti-HSV-1 antibody, and immunohistochemistry was performed. Specific HSV-1 staining is shown in brown. Representative images from each mouse group were chosen for the panels.

viral titer was brought down to 5.2 PFU. Similar to results for R3616, H9813 was unable to reach detectable levels in this tissue. In the brain (Fig. 2C), HSV-1(F) had average viral titers of 1.5, 4.2, and 3.5×10^1 PFU, respectively, on days one, three, and seven. In contrast, neither R3616 nor H9813 was detectable in the brain. Therefore, subtle mutations in $\gamma_1$34.5 prevented viral replication in the peripheral tissue and subsequent spread to the central nervous system. Our results provided evidence that V^{193} and F^{195} of $\gamma_1$34.5 indeed were essential for HSV neuroinvasion in vivo.

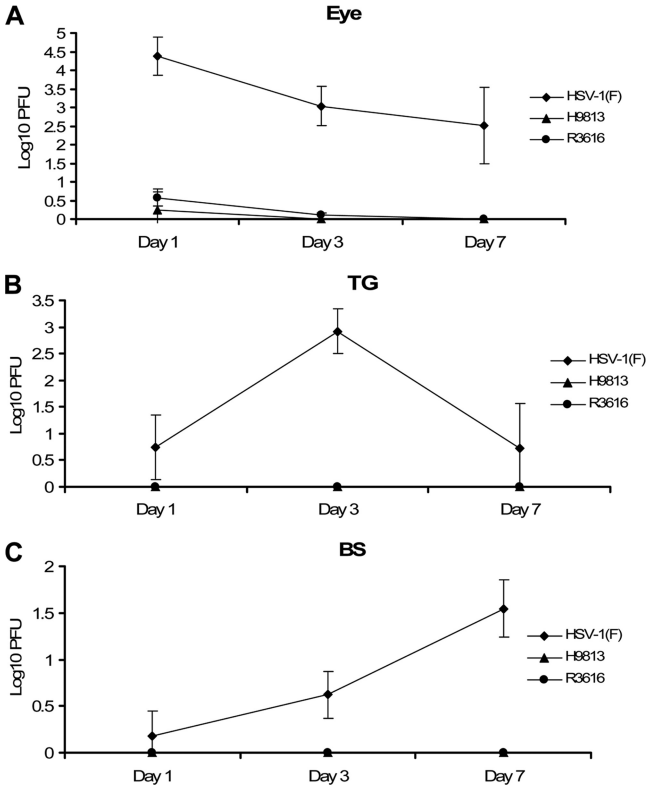

FIG. 2. Kinetics of viral replication in vivo. Groups of 6-week-old female BALB/c mice were mock infected or infected with HSV-1(F), R3616, or H9813 via bilateral corneal scarification with 4×10^5 PFU of virus. Mice from each group were sacrificed on days 1, 3, 5, and 7. At this time, tissue samples from the eye (A), trigeminal ganglia (TG) (B), or brain stem (BS) (C) were used for titration on Vero cells. Data are expressed as means ± standard deviations for five mice for each group.

It has been reported that in response to HSV infection, a number of antiviral mechanisms operate in a cell type- and time-dependent manner (24). Remarkably, TLR3 and the cytosolic sensors RIG-I, MDA5, and DAI relay signals to induce the interferon response (15, 23, 31). In this process, TBK1 activates IRF3 to induce antiviral responses. In addition, PKR, whose expression is elevated by interferon, is activated during HSV infection (6). To further characterize $\gamma_1$34.5, we examined viral growth in different mammalian cell lines. Cells were infected with HSV-1(F), R3616, or H9813 (0.05 PFU/ml), and virus yields were determined. The results displayed in Fig. 3A show that HSV-1(F) replicated to a titer of 3.0×10^6 PFU/ml 24 h after infection in SK-N-SH human neural cells. High levels of viral replication were maintained at 48 h, reaching 3.0×10^7 PFU/ml. Replication of R3616 was drastically reduced, with titers of 3.6×10^3 PFU/ml and 5.8×10^3 PFU/ml at 24 and 48 h, respectively. H9813 replicated similarly to R3616 over the same time period, reaching titers of 5.4×10^3 PFU/ml at 24 h and 1.2×10^4 PFU/ml at 48 h. Similar growth patterns were observed in CV-1 kidney epithelial cells (Fig. 3B), 3T6 mouse embryonic fibroblasts (Fig. 3C), and human embryonic lung (HEL) fibroblasts (Fig. 3D). In each case, H9813 replicated poorly, mirroring the growth patterns of R3616 virus. Thus, V^{193} and F^{195} in $\gamma_1$34.5 are required to overcome the inhibitory effect of host cells on HSV replication. Given that valine and phenylalanine in the PP1 binding motif are conserved among other PP1 binding proteins (9), these results suggest that the PP1 binding motif in $\gamma_1$34.5 may represent a functional module which dictates the outcome of HSV infection and pathogenesis.

To define the mechanistic basis of $\gamma_1$34.5 action, we analyzed viral growth and response to type I interferon (IFN) in Vero cells, which are deficient in alpha/beta IFN (IFN-α/β) and

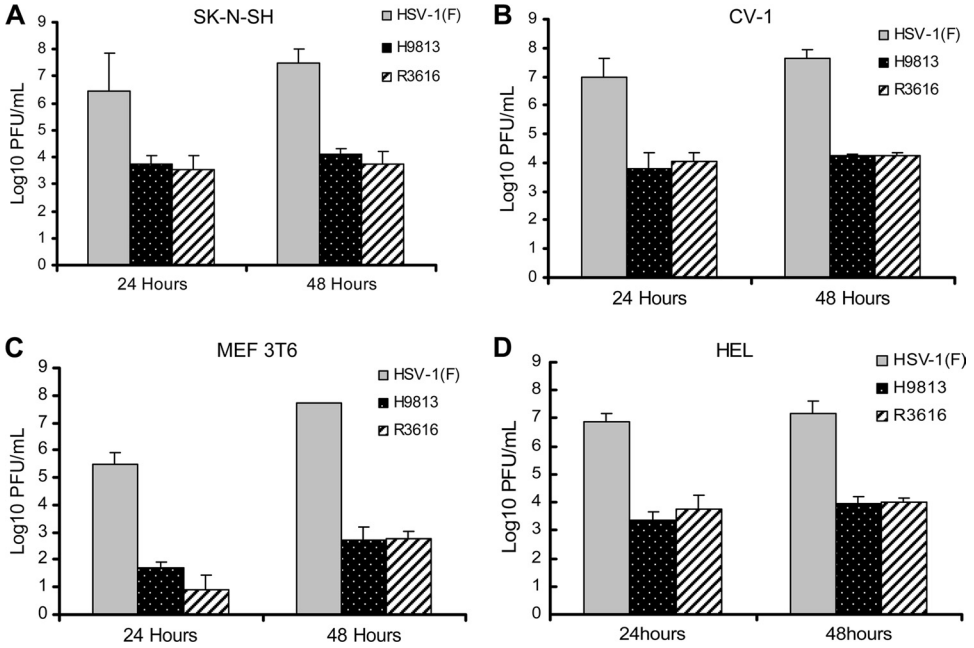

FIG. 3. Viral growth properties in mammalian cell lines. Confluent monolayers of SK-N-SH (A), CV-1 (B), MEF 3T6 (C), or HEL (D) cells were infected with HSV-1(F), R3616, or H9813 at 0.05 PFU per cell and incubated at 37°C. Viruses were harvested at 24 and 48 h postinfection. Samples were then freeze-thawed three times and titrated on Vero cells. Representative experiments done in triplicate are shown.

IRF3 production (5, 10). Cells, untreated or pretreated with IFN-α (200 U/ml), were infected with viruses at 0.05 PFU, and viral yields were determined. As shown in Fig. 4A, in the absence of IFN all viruses replicated efficiently, with titers ranging from 2.3×10^5 to 2.3×10^6 PFU/ml. $V^{193}E$ and $F^{195}L$ substitutions in $\gamma_1$34.5 or removal of $\gamma_1$34.5 had a marginal effect on HSV replication. This phenotype likely resulted from a defect in type I IFN or IRF3 production in Vero cells. When treated with type I IFN, only wild-type HSV-1(F) was able to replicate efficiently, reaching a titer of 6.2×10^5 PFU/ml. In sharp contrast, R3616 and H9813 viruses were highly sensitive to IFN treatment, with a titer reduced to 2.8×10^3 PFU/ml. Thus, the PP1 binding motif contributes to HSV resistance to type I IFN.

In HSV-infected cells, $\gamma_1$34.5 recruits PP1 to form a high-molecular-weight complex that dephosphorylate eIF2α (13). We evaluated eIF2α phosphorylation and viral protein synthesis. HEL fibroblasts were mock infected or infected with viruses. At 18 h postinfection, lysates of cells were subjected to Western blot analysis with antibodies against eIF2α, phosphor-

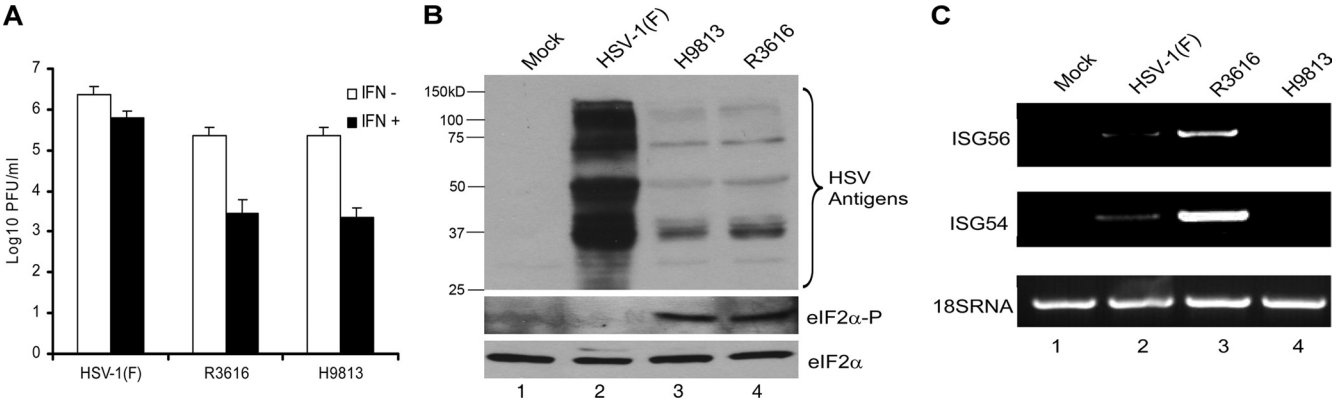

FIG. 4. (A) Viral response to IFN. Monolayers of Vero cells were either untreated or pretreated with IFN-α (200 U/ml; Sigma) for 20 h. Cells were then infected with indicated viruses at 0.05 PFU per cell and incubated at 37°C. At 24 h postinfection, cells were harvested and freeze-thawed three times, and virus yields were determined by titration. (B) Synthesis of viral proteins and eIF2α dephosphorylation. Monolayers of HEL fibroblasts were either mock infected or infected with viruses as indicated (5 PFU/cell). At 18 h after infection, lysates of cells were subjected to electrophoresis and reacted with antibodies against mixed HSV-1 antigens (Dako Coporation), eIF2α, and phosphorylated eIF2α (Cell signaling Tech). Size markers are listed on the left. (C) Viral induction of antiviral genes. HEL fibroblast cells were mock infected or infected with HSV-1(F), R3616, or H9813 (5 PFU/cell) as indicated. At 6 h postinfection, total RNA was extracted from cells and subjected to reverse transcription-PCR amplification and electrophoresis for ISG56, ISG54, and 18s rRNA.

ylated eIF2α, and HSV antigens. As indicated in Fig. 4B, HSV-1(F)-infected cells exhibited efficient viral polypeptide synthesis without detectable eIF2α phosphorylation. In contrast, both H9813- and R3616-infected cells had little viral protein synthesis, which paralleled increased eIF2α phosphorylation. Hence, Val^{193} and F^{195} in $\gamma_1$34.5 mediate eIF2α dephosphorylation, which is linked to viral protein synthesis and resistance to IFN-α/β.

Because $\gamma_1$34.5 also blocks the induction of antiviral genes by targeting TBK1 (26), we asked whether the interaction of $\gamma_1$34.5 with PP1 contributed to this process during HSV infection. HEL fibroblasts were mock infected or infected with viruses. At 6 h postinfection, reverse transcription-PCR analysis was performed to evaluate the expression of ISG54, ISG56, and 18S rRNA. As seen in Fig. 4C, R3616 significantly increased both ISG54 and ISG56 mRNA compared to HSV-1(F). H9813 was able to block the induction of these antiviral genes. There was no detectable accumulation of ISG54 or ISG56 mRNA. Consistently, like wild-type virus, H9813 also inhibited IRF3 nuclear translocation of IRF3, whereas R3616 stimulated IRF3 nuclear translocation (data not shown). Herein, perturbation of Val^{193} and F^{195} did not affect the ability of $\gamma_1$34.5 to block the induction of antiviral genes. We conclude that $\gamma_1$34.5 inhibits the induction of antiviral genes mediated by TBK1 independently of PP1. In this regard, it is interesting that H9813 was unable to replicate in the eye, trigeminal ganglia, and brain. This phenotype correlated with defective viral growth, eIF2α dephosphorylation, and resistance to IFN-α/β. Thus, suppression of virus-induced antiviral gene expression alone did not restore viral virulence. HSV $\gamma_1$34.5 appears to function in a temporal manner (8, 26). At the early stage of infection, it functions to inhibit or alleviate the induction of antiviral genes. As virus infection proceeds, it precludes eIF2α phosphorylation triggered by the onset of viral DNA replication. We speculate that $\gamma_1$34.5 may promote viral virulence in vivo by coordinately modulating PP1 and TBK1. Consistent with this idea, we demonstrate that eIF2α dephosphorylation by $\gamma_1$34.5 is essential to display HSV virulence.

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