Herpes Simplex Virus 1 ICP0 Phosphorylation Site Mutants Are Attenuated for Viral Replication and Impaired for Explant-Induced Reactivation

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Received 13 July 2011/Accepted 9 September 2011

In cell culture experiments, phosphorylation appears to be a critical regulator of the herpes simplex virus 1 (HSV-1) immediate-early (IE) protein, ICP0, which is an E3 ubiquitin ligase that transactivates viral gene expression. Three major regions of phosphorylation in ICP0 (amino acids 224 to 232, 365 to 371, and 508 to 518) have been identified, and mutant viruses that block phosphorylation sites within each region (termed Phos 1, 2, and 3, respectively) have been constructed. Previous studies indicated that replication of Phos 1 is significantly reduced compared to that of wild-type virus in cell culture (C. Boutell, et al., J. Virol. 82:10647–10656, 2008). To determine the effects these phosphorylation site mutations have on the viral life cycle in vivo, mice were ocularly infected with wild-type HSV-1, the Phos mutants, or their marker rescue counterparts. Subsequently, viral replication, establishment of latency, and viral explant-induced reactivation of these viruses were examined. Relative to wild-type virus, Phos 1 eye titers were reduced as much as 7- and 18-fold on days 1 and 5 postinfection, respectively. Phos 2 eye titers showed a decrease of 6-fold on day 1 postinfection. Titers of Phos 1 and 2 trigeminal ganglia were reduced as much as 16- and 20-fold, respectively, on day 5 postinfection. Additionally, the reactivation efficiencies of Phos 1 and 2 were impaired relative to wild-type HSV-1, although both viruses established wild-type levels of latency in vivo. The acute replication, latency, and reactivation phenotypes of Phos 3 were similar to those of wild-type HSV-1. We conclude from these studies that phosphorylation is likely a key modulator of ICP0’s biological activities in a mouse ocular model of HSV-1 infection.

A hallmark of herpes simplex virus 1 (HSV-1) infection is its capacity to establish both lytic and latent infections in its host. HSV-1 lytic infections, either primary infections or those due to reactivation, can lead to the development of oral-facial and ocular sores (56). Latent infection differs from lytic infection in that viral transcription is largely repressed, with the exception of the latency-associated transcripts (63); consequently, no infectious virus is produced (63). The first viral genes to be expressed during lytic infection are the immediate-early (IE) genes (37), which regulate viral and host gene expression and impair the host immune response. HSV-1 synthesizes five IE proteins, known as infected cell proteins (ICPs) 0, 4, 22, 27, and 47. Of these IE proteins, ICP0 is known to be required for efficient viral gene expression, replication, and reactivation from latency (9, 18, 31, 32, 39, 57, 64).

ICP0’s ability to promote viral replication is closely associated with its RING-finger motif, which possesses E3 ubiquitin ligase activity (8, 27), adding chains of ubiquitin to cellular substrates (5, 7), marking them for degradation by the 26S proteasome complex. ICP0’s E3 ubiquitin ligase activity enhances viral transcription (10, 11, 19, 26, 55) and disrupts the subnuclear structure known as nuclear domain 10 (ND10) (46, 47). ND10 and several of its constituent proteins, the promyelocytic leukemia protein (PML), Sp100, hDaxx, and ATRX, have been shown to limit HSV replication (21, 22, 52). Specifically, ICP0’s E3 ubiquitin ligase activity promotes the disruption of ND10 by mediating the proteolysis (directly or indirectly) of SUMO-conjugated isoforms of PML and Sp100 (12, 20, 50) or by dissociating the ND10 components hDaxx and ATRX (45), stimulating viral transcription. In addition to affecting viral transcription, ICP0 assists HSV-1 in counteracting other intrinsic and innate host defenses that include chromatinization or repression of the viral genome (13, 14, 23, 24, 28) and activation and establishment of the interferon response (35, 40, 48, 49, 54).

ICP0 is known to be phosphorylated, a posttranslational modification that acts to modulate the activities of many viral and cellular proteins (1, 68). Cellular cyclin-dependent kinases (cdks) and the viral kinase UL13 have been reported to phosphorylate ICP0 or to be required for maximal levels of ICP0 phosphorylation in the infected cells (3, 15, 53). The activities of cdks appear to modulate the biological properties of ICP0,
including its transactivating activity (15, 17). To directly determine the role phosphorylation plays in ICP0 functions, we mapped three phosphorylated regions in ICP0 and mutated phospho-acceptor sites in each of these regions, creating the mutants Phos 1, 2, and 3 (16). In transient expression assays, these phosphorylation site mutations affected several properties of ICP0, including its subcellular localization, E3 ubiquitin ligase activity, ability to dissociate proteins from ND10, and transactivation activity (6, 16). When these mutations were introduced into the HSV-1 genome, only Phos 1 was impaired for HSV-1 replication in cell culture (6). The purpose of the present study is to understand the impact these mutations have on HSV-1 acute replication, latency, and explant-induced reactivation in vivo. Consequently, the Phos mutants were tested in the mouse ocular model of HSV-1 latency and reactivation. Results from this study show that mutations in regions I and II of ICP0 affect acute replication and explant-induced reactivation, suggesting that phosphorylation regulates ICP0's biological functions in vivo.

MATERIALS AND METHODS

Cell lines and viruses. Vero cells (African green monkey kidney cell line) and L7 cells (Vero cells that contain the ICP0 gene) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS), 100 μg/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine as previously described (59, 60), except 5% fetal bovine serum (FBS) was used in our experiments. The wild-type HSV-1 strain KOS, 7134 (an ICP0-null mutant virus), Phos 1, 2, and 3, and their respective marker rescue viruses (Phos 1, 2, and 3 MR) were propagated and their titers determined as previously described (6, 11, 60).

Ocular infection of mice. CD-1 outbred female mice (6 to 7 weeks old) were obtained from Charles Rivers Laboratories (Shrewsbury, MA) and cared for according to the Guide for the Care and Use of Laboratory Animals (51), and the protocol for their use was approved by University of Kansas Institutional Animal Care and Use Committee. Mice were infected as previously described (4). Briefly, mice were anesthetized by intraperitoneal injection with ketamine (100 mg/kg of body weight) and xylazine (6.6 mg/kg of body weight). Corneas were scarified with a 26-gauge needle and infected with one of 8 viruses (KOS, 7134, Phos 1, Phos 2, Phos 3, Phos 1 MR, Phos 2 MR, or Phos 3 MR) at 2 × 10^4 PFU per eye in 3 μl medium. Mortality rates in our experiments (from day 0 to 30 days postinfection) for all viruses tested ranged from 0 to 19%.

Determination of viral titers in eyes and TG. On indicated days 0 to 9 postinfection, eye swabs and trigeminal ganglion (TG) samples were collected. For eye swabs, a sterile cotton-tipped swab was moistened with Vero cell medium. Tear film was collected by swabbing each eye and then placing the swab in 500 μl of Vero cell medium. The cultures were sampled daily (150 μl per sample) up to 16 days for the presence of infectious virus by cytopathic effect on Vero cell monolayers (for KOS and Phos 1, Phos 2, and Phos 3) or L7 cell monolayers (for 7134 and Phos 1, 2, and 3). Vero cell medium (300 μl/well) was added to each explant culture every other day. After taking samples on day 10 postexplantation, cultures were heat shocked at 43°C for 3 h to provide an added stimulus for reactivation. Statistically significant differences for viral reactivation were determined using the Fisher's exact test.

RESULTS

Mutations in Phos 1 and 2 impair viral replication in the eyes. We have previously shown that cluster mutations in one of three phosphorylated regions of ICP0 (Fig. 1) altered several properties of the protein, and at least one ICP0 phosphorylation mutant (Phos 1) was impaired for viral replication in cell culture (6, 16). To determine whether the relative replication efficiencies of the Phos mutant viruses were altered in vivo, we used the mouse ocular model of HSV latency and reactivation. Initially, to examine acute replication at the periphery, CD-1 mice were infected with 2 × 10^5 PFU of virus per eye, and eye swabs were taken up to 9 days postinfection. Viral titers were determined by standard plaque assay. For wild-type HSV-1 (KOS), viral replication in the eyes was biphasic (Fig. 2), peaking on days 1 and 5 postinfection, which has been observed in other studies (30, 33, 58); this increase in ocular replication on day 5 is likely attributed to viral spread from the
TG back to the eye (66). The replication of Phos 1 was reduced relative to that of KOS, on days 1 and 5 postinfection, with a 7-fold reduction ($P < 0.047$) on day 1 and an 18-fold reduction ($P < 0.01$) on day 5 (Fig. 2A). Interestingly, Phos 2 replication was only diminished 6-fold ($P < 0.039$) on day 1 postinfection. In contrast to the first two mutants, the replication of Phos 3 was comparable to that of KOS on all days tested (Fig. 2A). In the case of Phos 1 and 2, their replication was not as reduced as that of the ICP0 null mutant, 7134, which reached a 64-fold decrease on day 5 postinfection. As expected, the marker rescue (MR) viruses (Phos 1 MR, Phos 2 MR, and Phos 3 MR) replicated at levels similar to KOS (Fig. 2B). Collectively, these data indicate that the mutations in Phos 1 and 2 impair viral replication in the eyes.

Phos 1 and 2 are diminished for acute replication in the TG. To determine if the Phos mutations affected productive infection in the sensory neurons, viral replication of the Phos mutant viruses was monitored in the TG of ocularly infected mice. For TG titers on days 3 and 5 postinfection, Phos 1 replication was diminished 7-fold ($P < 0.01$) and 16-fold ($P < 0.00009$), respectively, compared to KOS replication (Fig. 3A). In the case of Phos 2, viral titers were reduced 18-fold day 3 postinfection ($P < 0.04$) and 20-fold day 5 postinfection ($P < 0.0002$) (Fig. 3A). Decreases in the TG viral titers of Phos 1 and Phos 2, however, did not approach those of 7134, which were reduced as much as 2,000-fold on days 3 to 7 postinfection. The acute replication of Phos 3, however, showed no significant difference compared with the wild-type virus (Fig. 3A). Titers of the Phos MR viruses were comparable to those of KOS (Fig. 3B). Our results show that the Phos 1 and 2 mutations impair acute viral replication in the TG, whereas the Phos 3 mutation does not influence the viral growth in the TG.

The Phos mutants are not impaired in establishing a latent infection. To quantify the amount of viral DNA present in latently infected neurons, TG were collected 30 days postinfection and assayed for the presence of HSV-1 DNA by real-time PCR. As shown in Fig. 4, the relative amounts of latent viral DNA present in TG were comparable to those of KOS for all of the Phos mutants. The ICP0 null mutant, 7134, was the only virus that showed a significant reduction (11-fold; $P < 0.01$), respectively, compared to KOS replication (Fig. 3A). In the case of Phos 2, viral titers were reduced 18-fold day 3 postinfection ($P < 0.04$) and 20-fold day 5 postinfection ($P < 0.0002$) (Fig. 3A). Decreases in the TG viral titers of Phos 1 and Phos 2, however, did not approach those of 7134, which were reduced as much as 2,000-fold on days 3 to 7 postinfection. The acute replication of Phos 3, however, showed no significant difference compared with the wild-type virus (Fig. 3A). Titers of the Phos MR viruses were comparable to those of KOS (Fig. 3B). Our results show that the Phos 1 and 2 mutations impair acute viral replication in the TG, whereas the Phos 3 mutation does not influence the viral growth in the TG.

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0.05) in viral DNA levels relative to KOS, and this reduction corresponded with previously published studies (31, 32). As expected, there were no appreciable differences in the latent viral genome loads among Phos 1 MR, Phos 2 MR, Phos 3 MR, and KOS.

Phos 1 and 2 show reduced efficiencies of explant-induced reactivation from latency. The efficiencies and kinetics of explant-induced reactivation from latency were established for all viral groups by collecting latently infected TG 30 days postinfection and culturing them ex vivo. Samples were examined daily for cytopathic effect, which indicates reactivation of virus from latent TG, and the total percentage of reactivating samples per virus was determined. In our initial set of studies, we examined the rates of reactivation for KOS, 7134, and the Phos mutants. KOS began to reactivate on day 3 postexplant (p.e.) at 66% and reached 100% reactivation by day 5 p.e. (Fig. 5A). Compared with KOS, Phos 1 and 2 reactivated at reduced rates of 33% ($P = 0.0003$) and 50% ($P = 0.0009$), respectively, on day 5 p.e. (Fig. 5A). Phos 3 reactivated efficiently, achieving 85% ($P = 0.15$) reactivation efficiency on day 5 p.e., which was comparable to that of KOS (Fig. 5A). The ICP0 null mutant, 7134, reactivated on day 10 p.e. and after heat shock reached its highest levels (58.8%) on day 16 p.e. (Fig. 5A). Notably, the reactivation phenotypes of Phos 1 and 2 were intermediate to those of KOS and 7134. In a second set of studies, we examined the reactivation efficiencies of the Phos mutants relative to their MR viruses. The MR viruses behaved as expected, rapidly reactivating from days 2 to 5 p.e., eventually reaching efficiencies of 87 to 92% by day 5 p.e. (Fig. 5B). In contrast, Phos 1 reached a peak efficiency of 48% by day 11 p.e. ($P = 0.0002$), and Phos 2 peaked at 68% by day 11 p.e. ($P = 0.01$) (Fig. 5B). The Phos 3 reactivation rate resembled its MR virus with a rate of 85% on day 5 p.e. and reached 100% at the end of the study (Fig. 5B). These explant-induced reactivation studies show that the frequencies and kinetics of reactivation are altered for two of the Phos mutants, with Phos 1 showing the greatest deficiency followed by Phos 2.

**DISCUSSION**

In this study, we have shown that two ICP0 phosphorylation site mutants are impaired for acute replication and explant-induced reactivation using the mouse ocular model of HSV infection. Specifically, Phos 1 and 2 were impaired for acute ocular and neuronal replication and explant-induced reactivation from latency. Interestingly, all Phos mutants established latency at wild-type levels, even though the levels of Phos 1 and 2 acute replication in the TG were intermediate compared to those of wild-type HSV-1 and the ICP0 null mutant, 7134. Given that the degree of acute viral replication in the TG correlates with the establishment of latency (38, 39), the decreases in Phos 1 and 2 acute neuronal replication were, nonetheless, sufficient to allow these viruses to establish efficient latent infections. The life cycle of Phos 3 in mice mirrored wild-type HSV-1 in our experiments, indicating that phosphorylation of residues in region III of ICP0 (Fig. 1A) is not re-
duced for productive infection and explant-induced reactivation in mice. Of the Phos mutants examined, Phos 1 was the only mutant to show replication deficiencies in cell culture (6, 16) and in vivo, as demonstrated in this report. Notably, Phos 2 had no obvious viral growth defects in cell culture (6, 16); however, decreased levels of viral replication and explant-induced reactivation observed with Phos 2 only became apparent when tested in the mouse ocular model, highlighting the importance of characterizing our mutants in vivo. While we tested only one viral dose in this study, it is also possible that the replication, latency, and/or reactivation phenotypes of Phos 1, 2, and 3 may be greater or become evident when infecting mice with lower input viral doses (<2 × 10^5 PFU/eye). Overall, our results suggest that phosphorylation plays an important role in the biology of ICP0 to enhance HSV-1 acute replication in vivo and reactivation ex vivo. Studies are being conducted to identify the mechanisms responsible for the in vivo phenotypes of Phos 1 and Phos 2.

For Phos 1, its mutation lies in the proline-rich transactivation domain and is adjacent to the RING-finger domain of ICP0 (Fig. 1). We have previously shown that Phos 1 is impaired for ICP0's E3 ubiquitin ligase activity, dispersal of ND10-associated proteins, and transactivator function, in the absence of other viral factors, and has enhanced protein stability during viral infection (6). Thus, defects in Phos 1 autoubiquitination and its ubiquitination of other cellular target proteins are likely contributors to its reduced levels of acute replication and reactivation. The delay and reduced efficiency of explant-induced reactivation observed with Phos 1 could also be linked to alterations in viral chromatinization and apoptosis. ICP0 is known to enhance viral gene expression during reactivation, which is associated with a decrease in the chromatinization of the viral genome (14, 24). Indeed, unlike wild-type strains of HSV-1, a RING-finger mutant of ICP0, which is impaired for ICP0's E3 ubiquitin ligase activity, was unable to induce viral gene expression in quiescently infected cultures (25). Because Phos 1 is defective or diminished for ubiquitin conjugation, Phos 1 may not be as proficient as wild-type ICP0 in altering the structure of viral chromatin to promote viral transcription and replication. Lastly, ICP0 has been shown to ubiquitinate the tumor suppressor protein, p53, and limit apoptosis in virally infected cells upon DNA damage (7).

Thus, Phos 1 may not efficiently mediate the degradation of p53 or other antiapoptotic targets during reactivation, potentially increasing apoptotic signaling, thereby reducing the levels and kinetics of reactivation. Clearly, the impairment of these activities does not impede Phos 1's ability to establish an efficient latent infection, which is similar to that of KOS.

The Phos 2 mutation, which is also located in a proline-rich transactivation domain of ICP0 (Fig. 1), has a minor defect in ocular shedding and is significantly defective in neuronal replication and explant-induced reactivation from latency. These results indicate that phosphorylated residues in region II of ICP0 are important for efficient viral replication, especially in sensory neurons. One potential explanation for Phos 2's attenuation in vivo is its ability to differentially affect the dissociation of cellular proteins from ND10. The Phos 2 mutant form of ICP0 is unable to dissociate PML from ND10 but is able to dissociate Sp100 from ND10 in a cell-type-dependent manner (6). Thus, the inability of Phos 2 to alter the subcellular localization of PML, which is a part of the host’s antiviral response, could diminish its viral replication. Related to its ND10-disrupting activity, ICP0 has been described to contain a SUMO-interacting motif (SIM) in region II (5a). A SIM facilitates interactions with SUMO isoforms or SUMO-conjugated proteins (reviewed in references 34 and 36). A recent publication showed that phosphorylation of SIM domains in at least three cellular proteins, including PML, significantly enhances their interaction with SUMO isoforms (62). Thus, in the case of Phos 2, blocking phosphorylation in region II may impede this putative SUMO-binding activity, affecting its interaction with SUMO-conjugated proteins (such as PML) or specific SUMO isoforms. Lastly, the Phos 2 mutation lies within a region that contains Src homology 3 (SH3) binding motifs, which allow for interaction with a subset of Src kinase family members and other SH3-containing proteins (41, 42). These kinases and factors play important roles in cell signaling. Thus, Phos 2 might interrupt these interactions in neurons, impairing acute replication and explant-induced reactivation.

Of the three Phos mutants, only mutations in Phos 3 did not influence the replication of HSV-1 in vivo, similar to our previously published results with cell culture (6, 16). While mutations in Phos 3 appear to affect its subcellular localization and transactivating activity when expressed in transient-transfection assays (16), our results indicate that the phenotypes observed with Phos 3 in cell-based assays do not impact acute infection and latency in vivo and explant-induced reactivation. How phosphorylation of region III in ICP0 contributes to HSV replication remains to be determined.

It has been demonstrated that the phosphorylation state of ICP0 is altered during the course of the viral replication cycle (1, 2, 15, 53, 68), suggesting that differential phosphorylation of ICP0 might activate or inhibit one or more specific functions of this key viral regulatory protein. Consequently, the identity of specific phosphorylation sites in regions I and II of ICP0, the potential kinases that phosphorylate these sites, and their temporal regulation and roles in ICP0's functions will likely elucidate new mechanisms in the switch between the lytic and latent infections of HSV-1.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant R01AI72357 from the National Institute of Allergy and Infectious Diseases (D.J.D.) and Public Health Service grant R01CA20260 from the National Cancer Institute (for P.A.S.).

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank members of the Davido lab, former members of the Schaffer lab, and Steve Benedict for helpful comments and suggestions. This article was written in honor of Priscilla Schaffer, who passed away late in 2009. Priscilla was an excellent virologist and a caring, dedicated mentor. Her enthusiasm for biology and scientific contributions to the field of herpesvirology will be greatly missed.

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simplex virus 1 is sequential and reflects the subcellular compartment in which the proteins localize. J. Virol. 75:7904–7912.


