

# Phosphorylation of a Herpes Simplex Virus 1 dUTPase by a Viral Protein Kinase, Us3, Dictates Viral Pathogenicity in the Central Nervous System but Not at the Periphery

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## ABSTRACT

Herpes simplex virus 1 (HSV-1) encodes Us3 protein kinase, which is critical for viral pathogenicity in both mouse peripheral sites (e.g., eyes and vaginas) and in the central nervous systems (CNS) of mice after intracranial and peripheral inoculations, respectively. Whereas some Us3 substrates involved in Us3 pathogenicity in peripheral sites have been reported, those involved in Us3 pathogenicity in the CNS remain to be identified. We recently reported that Us3 phosphorylated HSV-1 dUTPase (vdUTPase) at serine 187 (Ser-187) in infected cells, and this phosphorylation promoted viral replication by regulating optimal enzymatic activity of vdUTPase. In the present study, we show that the replacement of vdUTPase Ser-187 by alanine (S187A) significantly reduced viral replication and virulence in the CNS of mice following intracranial inoculation and that the phosphomimetic substitution at vdUTPase Ser-187 in part restored the wild-type viral replication and virulence. Interestingly, the S187A mutation in vdUTPase had no effect on viral replication and pathogenic effects in the eyes and vaginas of mice after ocular and vaginal inoculation, respectively. Similarly, the enzyme-dead mutation in vdUTPase significantly reduced viral replication and virulence in the CNS of mice after intracranial inoculation, whereas the mutation had no effect on viral replication and pathogenic effects in the eyes and vaginas of mice after ocular and vaginal inoculation, respectively. These observations suggested that vdUTPase was one of the Us3 substrates responsible for Us3 pathogenicity in the CNS and that the CNS-specific virulence of HSV-1 involved strict regulation of vdUTPase activity by Us3 phosphorylation.

## IMPORTANCE

Herpes simplex virus 1 (HSV-1) encodes a viral protein kinase Us3 which is critical for pathogenicity both in peripheral sites and in the central nervous systems (CNS) of mice following peripheral and intracranial inoculations, respectively. Whereas some Us3 substrates involved in Us3 pathogenicity in peripheral sites have been reported, those involved in Us3 pathogenicity in the CNS remain to be identified. Here, we report that Us3 phosphorylation of viral dUTPase (vdUTPase) at serine 187 (Ser-187), which has been shown to promote the vdUTPase activity, appears to be critical for viral virulence in the CNS but not for pathogenic effects in peripheral sites. Since HSV proteins critical for viral virulence in the CNS are, in almost all cases, also involved in viral pathogenicity at peripheral sites, this phosphorylation event is a unique report of a specific mechanism involved in HSV-1 virulence in the CNS.

Herpes simplex virus 1 (HSV-1) encodes at least two protein kinases, Us3 and UL13 (1–6). HSV-1 Us3 is a serine/threonine protein kinase with an amino acid sequence that is conserved in the subfamily *Alphaherpesvirinae* (1, 2). The Us3 protein and its enzymatic activity have been suggested to play a critical role in viral replication and pathogenicity, based on studies showing that recombinant Us3-null mutant viruses and recombinant viruses encoding catalytically inactive Us3 (Us3 kinase-dead mutant viruses) have impaired growth properties in cell cultures and reduced pathogenicity and replication in mouse models (7–11).

In experimental animal models of HSV-1 infection, viral pathogenicity in peripheral sites (e.g., eyes and vaginas) and in central nervous systems (CNS) was studied by peripheral and intracranial inoculations, respectively. These are semi-independent indicators of viral virulence since viral proteins or their domains that are important for viral pathogenicity in peripheral sites are often not required for pathogenicity in the CNS in mouse models of HSV-1 infection (12–15). HSV-1 Us3 has been reported to be critical for viral pathogenicity in both peripheral sites and in the CNS in mice, based on studies showing that Us3-null and Us3 kinase-dead mutant viruses had significantly reduced viral repli-

cation and pathogenicity in the eyes and brains of mice following ocular and intracranial inoculation, respectively (7, 10, 11).

HSV-1 Us3 has been considered to be a multifunctional protein regulating various aspects of cellular and viral functions (5). Supporting to this hypothesis, more than 15 putative viral and cellular substrates of Us3 have been reported (16–27). However, among them, only a limited substrates (18–28), including gB, UL31, Us3 itself, UL47, viral dUTPase (vdUTPase), and tuberous sclerosis complex 2, have been shown both to be physiological Us3 substrates in infected cells and directly linked with Us3 functions in infected cells (11, 15, 24–28). Among these functional Us3 sub-

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strates, gB, Us3 itself, and UL47 have been shown to be involved in pathogenicity in peripheral sites, based on data showing that amino acid substitutions in the Us3 phosphorylation sites of these substrates significantly impaired viral replication and pathogenic effects in the eyes of mice after ocular inoculation (11, 15, 25). In contrast, these mutations had no effect on viral pathogenicity in mice following intracranial inoculation (11, 15, 25). Thus, although mechanisms of Us3 pathogenicity in peripheral sites have been gradually elucidated, the mechanisms of Us3 pathogenicity in the CNS remain unknown at present.

We recently reported that Us3 phosphorylated vdUTPase at serine 187 (Ser-187) in infected cells (17). dUTPases are known to catalyze hydrolysis of dUTP to dUMP and pyrophosphate and play a role in accurate replication of DNA genomes by preventing the misincorporation of dUTP into replicating DNA (29–32). HSV-1 dUTPase (vdUTPase) is encoded by the UL50 gene and is conserved throughout the *Herpesviridae* family (33, 34). Us3 phosphorylation of vdUTPase at Ser-187 appeared to regulate optimal activity of vdUTPase in infected cells, based on the observations that an alanine replacement of vdUTPase Ser-187 (S187A), as well as a Us3 kinase-dead mutation, significantly reduced vdUTPase activity in infected cells, whereas a phosphomimetic substitution at vdUTPase Ser-187 restored the wild-type vdUTPase activity (17). Furthermore, replication of a recombinant virus carrying the S187A or enzyme-dead mutation in vdUTPase was significantly impaired in a human neuroblastoma SK-N-SH cells at a multiplicity of infection (MOI) of 5, but not at an MOI of 0.01, whereas replication in human carcinoma HEP-2 and simian kidney epithelial Vero cells was not affected (17). These observations suggested that Us3 phosphorylation of vdUTPase at Ser-187 promoted viral replication in a manner dependent on cell types and MOIs by regulating optimal enzymatic activity of vdUTPase (17). Notably, it has been reported that a recombinant HSV-1 carrying a null mutation in vdUTPase was less virulent than wild-type virus, replicated less well in the CNS and reactivated less efficiently in a mouse model of HSV-1 infection (35), suggesting that vdUTPase was critical for viral replication and pathogenicity *in vivo*. Taking this together with our recent report described above (17), it is reasonable to hypothesize that Us3 phosphorylation of vdUTPase at Ser-187 is involved in viral replication and pathogenicity *in vivo*, especially in the CNS. In the present study, to test this hypothesis, we examined the effects of this phosphorylation on viral replication and pathogenicity *in vivo* and presented the data showing that the phosphorylation was required for efficient viral replication and virulence in the CNS of mice following intracranial inoculation. Surprisingly, we have also shown that the phosphorylation was not required for pathogenicity at peripheral sites following inoculation at these sites, i.e., the eyes and vaginas of mice. Thus, Us3 phosphorylation of vdUTPase at Ser-187 appeared to be involved in the CNS-specific viral replication and virulence.

## MATERIALS AND METHODS

**Cells and viruses.** Vero and rabbit skin cells were described previously (36, 37), as was HSV-1 wild-type strain HSV-1(F) (38). A number of the recombinant viruses were described previously (17, 24): recombinant virus YK511, encoding an enzymatically inactive Us3 mutant in which lysine at Us3 residue 220 was replaced with methionine (Us3K220M); recombinant virus YK513, in which the Us3K220M mutation in YK511 was repaired (Us3K220M-repair); recombinant virus YK750, in which the vdUTPase gene was disrupted by insertion of a foreign gene cassette containing an I-SceI site, a kanamycin resistance

gene, and 37 bp of the vdUTPase sequence encoding codons 181 to 193 in which Ser-187 was replaced with alanine ( $\Delta$ vdUTPase); recombinant virus YK751, encoding vdUTPase with an alanine substituted for Ser-187, that was constructed by excision of a part of the foreign gene cassette, including the I-SceI site and the kanamycin resistance gene in YK750 vdUTPase gene (vdUTPaseS187A); recombinant virus YK752, in which the vdUTPaseS187A mutation in YK751 was repaired (vdUTPase $\Delta$ /SA-repair); recombinant virus YK753 with the vdUTPaseS187D mutation (vdUTPaseS187D); recombinant virus YK754, in which the vdUTPaseS187D mutation in YK753 was repaired (vdUTPaseS187D-repair); recombinant virus YK759, encoding an enzymatically inactive vdUTPase mutant in which aspartic acid at vdUTPase residue 97 was replaced with alanine (vdUTPaseD97A); and recombinant virus YK760, in which the vdUTPaseD97A mutation in YK759 was repaired (vdUTPaseD97A-repair). We note that YK752 (vdUTPase $\Delta$ /SA-repair) can be the repaired virus for both YK750 ( $\Delta$ vdUTPase) and YK751 (vdUTPaseS187A), based on the sequential construction strategy of the recombinant viruses as described above and previously (17).

**Mutagenesis of viral genomes and generation of recombinant HSV-1.** Recombinant viruses YK755 encoding ICP22 with alanines substituted for Ser-5, Ser-22, Thr-162, and Ser-167 (ICP22S5A/S22A/T162A/S167A) (Fig. 1), and YK757 encoding UL51 with alanine substituted for threonine at residue Thr-190 (UL51T190A) (Fig. 1) were generated by the two-step Red-mediated mutagenesis procedure using *Escherichia coli* GS1783 containing pYbac102 as described previously (24, 39), except using the primers listed in Table 1. Recombinant viruses YK756 and YK758 in which the ICP22S5A/S22A/T162A/S167A and UL51T190A mutations in YK755 and YK757, respectively (Fig. 1), were repaired were generated as described previously (24, 39), except using the primers listed in Table 1 and pBS-ICP22-Kan (40).

**Animal studies.** Female ICR mice were purchased from Charles River. For intracranial infection, 3-week-old mice were infected intracranially with  $10^2$  PFU of each of the indicated viruses as described previously (36). Mice were monitored daily, and mortality from 1 to 14 days postinfection was attributed to the inoculated virus. Virus titers in the brains of mice were determined as described previously (41). For ocular infection, 5-week-old mice were infected with  $2 \times 10^6$  PFU/eye of each of the indicated viruses as described previously (11). The scoring scale for the severity of herpes stromal keratitis (HSK) was described previously (11). Virus titers in the tear films of mice were determined as described previously (11). For intravaginal infection, 4-week-old mice were each injected subcutaneously in the neck ruff with 1.67 mg of medroxyprogesterone (Depo-M; Vesco) in 100  $\mu$ l at 7 days prior to viral infection. The treated mice were then infected intravaginally with  $5 \times 10^6$  PFU of each of the indicated viruses (42). Mice were monitored daily until 14 days postinfection for survival and the severity of vaginal disease using a scoring system of 0 (no sign of disease), 1 (slight genital erythema and edema), 2 (moderate genital inflammation), 3 (purulent genital lesions), 4 (hind-limb paralysis), and 5 (death) as described previously (42). Virus titers in the vaginal secretions of mice were determined as described previously (42). All animal experiments were carried out in accordance with the Guidelines for Proper Conduct of Animal Experiments, Science Council of Japan. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Medical Science, The University of Tokyo (IACUC protocol approval 19-26).

**Statistical analysis.** Differences in virus titers, HSK scores, and vaginal lesion scores were statistically analyzed by using the two-tailed Student *t* test. Differences in mortality of infected mice were statistically analyzed by using a log-rank test.

## RESULTS

**Effect of phosphorylation of vdUTPase Ser-187 on viral replication and virulence in the CNS of mice.** To investigate the relevance of phosphorylation of vdUTPase Ser-187 in viral repli-

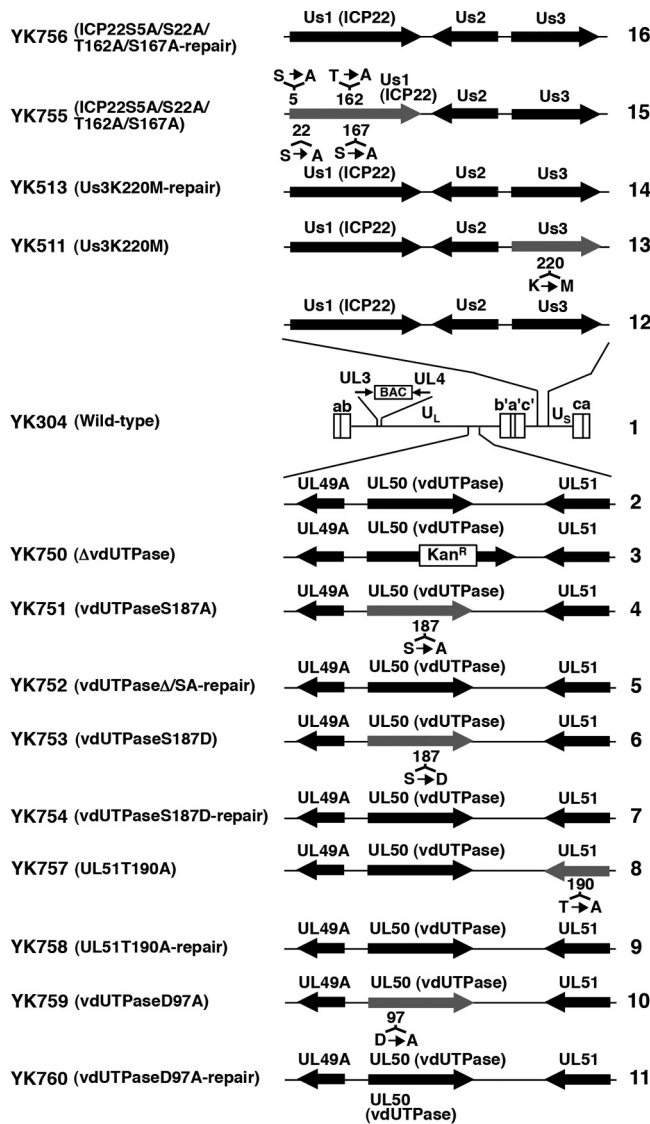


FIG 1 Schematic diagrams of the genome structure of wild-type and recombinant viruses used in the present study. Line 1, Wild-type HSV-1 (YK304) genome carrying a bacmid (BAC) in the intergenic region between UL3 and UL4. Line 2, domain carrying the UL49A, UL50 (vdUTPase) and UL51 open reading frames. Lines 3 to 7, 10, and 11, recombinant viruses with a mutation in the UL50 (vdUTPase) gene. Lines 8 and 9, recombinant viruses with a mutation in the UL51 gene. Lines 13 and 14, recombinant viruses with a mutation in the Us3 gene. Lines 15 and 16, recombinant viruses with mutations in the Us1 (ICP22) gene.

cation and virulence in the CNS of mice, 3-week-old female ICR mice were inoculated intracranially with  $10^2$  PFU of YK750 ( $\Delta$ vdUTPase), YK751 (vdUTPaseS187A), or YK511 (Us3K220M) or the repaired viruses YK752 (vdUTPase $\Delta$ /SA-repair) and YK513 (Us3K220M-repair). The survival of the infected mice was monitored for 14 days postinfection, and virus titers in infected mouse brains were assayed at 3 days postinfection. As shown in Fig. 2A and B, all or most of the mice infected with YK750 ( $\Delta$ vdUTPase), YK511 (Us3K220M), or YK751 (vdUTPaseS187A) survived, but the repaired viruses YK752 (vdUTPase $\Delta$ /SA-repair) and YK513 (Us3K220M-repair) killed 68 to 75% of infected mice. These results are in agreement with previous reports (10, 11, 35) that the null mutation in vdUTPase and

the kinase-dead mutation in Us3 significantly reduced mortality in mice after intracranial inoculation. Similarly, the vdUTPase S187A mutation also significantly reduced mortality in mice (Fig. 2A and C), indicating that vdUTPase Ser-187 was required for efficient virulence in the CNS of mice.

To further investigate the linkage between phosphorylation of vdUTPase Ser-187 and virulence in the CNS of mice, we also studied the virulence of YK753 (vdUTPaseS187D) carrying a phosphomimetic mutation in vdUTPase Ser-187 and its repaired virus YK754 (vdUTPaseS187D-repair) and of YK751 (vdUTPaseS187A) and its repaired virus YK752 (vdUTPase $\Delta$ /SA-repair) in the CNS of mice. As shown in Fig. 2C, after intracranial inoculation, the survival of mice infected with YK753 (vdUTPaseS187D) was significantly less than that of mice infected with YK751 (vdUTPaseS187A), and the survival of mice infected with YK752 (vdUTPase $\Delta$ /SA-repair) or YK754 (vdUTPaseS187D-repair) was even less than that of mice infected with YK753 (vdUTPaseS187D). This suggested that a negatively charged amino acid at vdUTPase Ser-187, due to either the phosphorylation of Ser-187 or an S187D substitution, was required for efficient virulence in the CNS of mice. Furthermore, in agreement with the effects of null, S187A, and S187D mutations in vdUTPase, and the kinase-dead mutation in Us3 on the survival of mice following intracranial inoculation, the null and S187A mutations in vdUTPase, and the kinase-dead mutation in Us3 significantly reduced viral replication in the brains of mice (Fig. 3A and B), whereas the S187D mutation in vdUTPase had little effect (Fig. 3C). Taken together, these results suggested that Us3 phosphorylation of vdUTPase Ser-187 was required for efficient viral virulence and replication in the CNS of mice following intracranial inoculation.

**Effect of phosphorylation of vdUTPase Ser-187 on viral replication and pathogenicity at peripheral sites in mice.** We previously reported that HSV-1 Us3 kinase activity is critical for development of herpes stroma keratitis (HSK) and viral replication in the eyes of mice after ocular inoculation (11). Us3 kinase activity is also critical for development of pathogenicity and viral replication in the vaginas of mice after vaginal inoculation, based on the observation that the Us3 kinase-dead mutation significantly reduced the pathogenicity scores of vaginal lesions and the virus titers in infected vaginas (Fig. 4A and 5A).

To examine whether the phosphorylation of vdUTPase Ser-187 was also involved in HSV-1 pathogenicity at peripheral sites, we carried out two series of experiments. In the first series of experiments, 5-week-old female ICR mice were infected ocularly with  $2 \times 10^6$  PFU/eye of YK750 ( $\Delta$ vdUTPase), YK751 (vdUTPaseS187A), or YK752 (vdUTPase $\Delta$ /SA-repair). The infected mice were observed daily for development of HSK for 14 days postinfection, and virus titers in tear films were determined at 1 and 4 days postinfection. In agreement with the reduction in virulence of YK750 ( $\Delta$ vdUTPase) in mice following intracranial inoculation, mice ocularly infected with YK750 ( $\Delta$ vdUTPase) exhibited significantly reduced severity of HSK compared to mice infected with the repaired virus YK752 (vdUTPase $\Delta$ /SA-repair) (Fig. 4B), and virus titers in the tear films of mice infected with YK750 ( $\Delta$ vdUTPase) were significantly lower than those of mice infected with YK752 (vdUTPase $\Delta$ /SA-repair) (Fig. 5B). In contrast, although the pathogenicity of YK751 (vdUTPaseS187A) was significantly attenuated in mice following intracranial inoculation (Fig. 2A and C), mice infected with YK751 (vdUTPaseS187A) exhib-



TABLE 1 Primer sequences for the construction of recombinant viruses

Mutation	Orientation <sup>a</sup>	Sequence (5'–3')
ICP22S5A/S22A	F	CACTGTGGTCTCCGGGACGTTTTCTGGATGGCCGACATTGCCCCAGGCGCTTTGCGCCTTGTGTAAA AGCGCGGCGTCCCCTCTCCGAGCCAGGATGACGACGATAAGTAGGG
	R	GCGGGAAGGGCGCTTTCGCGTTGCGCGTGCCAGGGGCGGGGCTCGGAGAGCGGGACGCCGCGCTTT TACACAAGGCGCAAAGCGCCTGGGGCAACCAATTAACCAATTCTGATTAG
ICP22T162A/S167A	F	GCGGGTCCGGTCTACCCGGAAACGCAGCCCCGGGCCCCCGCCCGTTCGCCCCAGCCCCAAATGCAA TGAGGATGACGACGATAAGTAGGG
	R	TCTGGGCTGGCGACCCGAGCGCCGTAGCATTGCATTTGGGGCTGGGGCCGACGGGGCGGGGG CCCGGGCAACCAATTAACCAATTCTGATTAG
ΔICP22	F	GCGGGGGAAGCCACTGTGGTCTCCGGGACGTTTTCTGGGTCCGGTTCGCCCCAGCCCCAGGATG ACGACGATAAGTAGGG
	R	TTTTATTTGGGGACATACAAGGGGGTTCGGGGCGACCGGAC-CCAGAAAACGTCCCGGAGGACAACCAA TTAACCAATTCTGATTAG
ΔICP22-repair	F	GCGGGGGAAGCCACTGTGGTCTCCGGGACGTTTTCTGGATGGCCGACATTTCCCAGGCGC CTTTTATTTGGGGACATACAAGGGGGTTCGGGGCGACCGGACTCACGGCCGAGAAAACGTGT
	R	
UL51T190A	F	GCTTGGGGTGACCGAGGCGCCCTCCTTGGGGCACCCCCACGCACCGCCCCGGAGGTTACAGGATGAC GACGATAAGTAGGG
	R	CGTTTCGGGCGGCAGGCCAGCGTAACCTCCGGGGCGGTGCGTGGGGGTGCCCAAGGCAACCAA TTAACCAATTCTGATTAG
UL51T190A-repair	F	GCTTGGGGTGACCGAGGCGCCCTCCTTGGGGCACCCCCACGCACCGCCCCGGAGGTTACAGGATG ACGACGATAAGTAGGG
	R	CGTTTCGGGCGGCAGGCCAGCGTAACCTCCGGGGCGGCGTGTGGGGGTGCCCAAGGCAACCAA TTAACCAATTCTGATTAG

<sup>a</sup> F, forward; R, reverse.

ited a severity of HSK almost identical to mice infected with YK752 (vdUTPaseΔ/SA-repair) (Fig. 4B), and the virus titers in the tear films of mice infected with YK751 (vdUTPaseS187A) were similar to those of mice infected with YK752 (vdUTPaseΔ/SA-repair) (Fig. 5B).

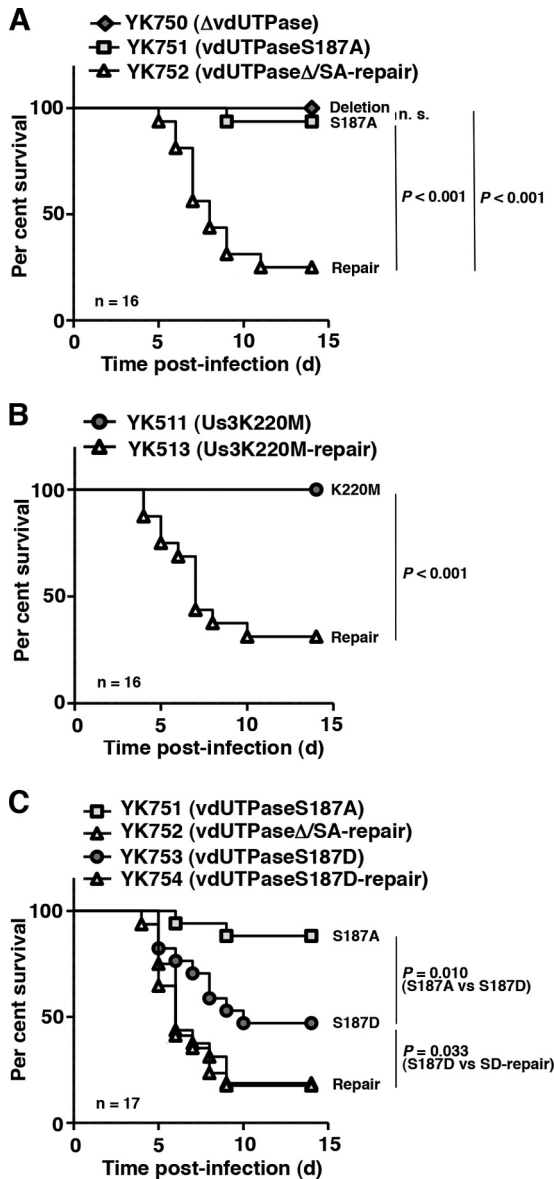
In the second series of experiments, 5-week-old female ICR mice pretreated with Depo-M were infected vaginally with  $5 \times 10^6$  PFU of YK750 (ΔvdUTPase), YK751 (vdUTPaseS187A), or YK752 (vdUTPaseΔ/S187A-repair). Disease progression was observed daily for 14 days postinfection, and the virus titers in vaginal secretions were determined at 1 and 3 days postinfection. In agreement with the results in the mouse HSK model, the null mutation in vdUTPase significantly reduced the development of vaginal disease and viral replication in mouse vaginas, whereas the vdUTPase S187A mutation had no significant effect on vaginal pathogenicity or viral replication (Fig. 4C and 5C).

Taken together, these results indicated that phosphorylation of vdUTPase Ser-187 was not required for HSV-1 pathogenicity and replication in the eyes and vaginas of mice following ocular and vaginal inoculation, respectively, although the vdUTPase protein itself was critical for efficient viral pathogenicity and replication in peripheral sites of mice.

**Effect of phosphorylation of other viral proteins on viral pathogenesis and replication in mice.** To confirm the specificity of the role of phosphorylation of vdUTPase Ser-187 in viral pathogenesis and replication in mice, we constructed recombinant viruses YK755 (ICP22S5A/S22A/T162A/S167A) and YK757 (UL51T190A), in which the phosphorylation sites in ICP22 and UL51 identified by phosphoproteomic analyses in previous studies (17, 43) were replaced with alanines, and their repaired viruses YK756 (ICP22S5A/S22A/T162A/S167A-repair) and YK758 (UL51T190A-repair)

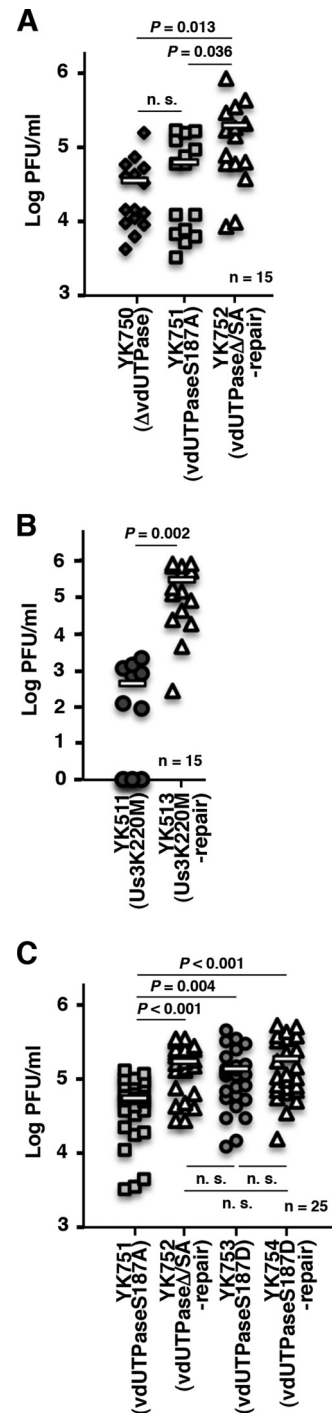
(Fig. 1), and used them to infect cultured cells and mice. Alanine substitutions for the phosphorylation sites in ICP22 and UL51 had no effect on viral growth in Vero cells at multiplicities of infection (MOIs) of 5 and 0.01, or on the survival of mice after intracranial inoculation (Fig. 6). These mutations also had no effect on viral replication and pathogenicity in the eyes of mice following ocular inoculation (Fig. 7A to F). These results indicated that the phosphorylation of ICP22 Ser-5, Ser-22, Thr-162, and Ser-167, and of UL51 Thr-190 was not required for viral replication and pathogenesis in mice after intracranial and ocular inoculation.

**Effect of vdUTPase enzymatic activity on viral replication and pathogenesis in mice.** Our previous and present observations that Us3 phosphorylation of vdUTPase Ser-187 was required for optimal vdUTPase enzymatic activity in infected cells (17) and for viral virulence in the mouse CNS (Fig. 2A and C) led us to hypothesize that vdUTPase enzymatic activity regulated viral virulence, in particular in the CNS of mice. To investigate this hypothesis, we examined the replication and pathogenicity of YK759 (vdUTPaseD97A) encoding an enzymatically inactive vdUTPase mutant in which vdUTPase Asp-97 was substituted with alanine and its repaired virus YK760 (vdUTPaseD97A-repair) in mice following intracranial, ocular, and vaginal inoculations. In agreement with the observations with YK751 (vdUTPaseS187A) and its repaired virus YK752 (vdUTPaseΔ/S187A-repair), following intracranial inoculation, there was a significant increase in mortality and progeny virus titers in the CNS of mice inoculated with YK760 (vdUTPaseD97A-repair) compared to mice inoculated with YK759 (vdUTPaseD97A) (Fig. 8A and 9A). In contrast, the level of HSK, vaginal disease and viral replication were similar in the eyes and vaginas of mice inoculated with either YK759

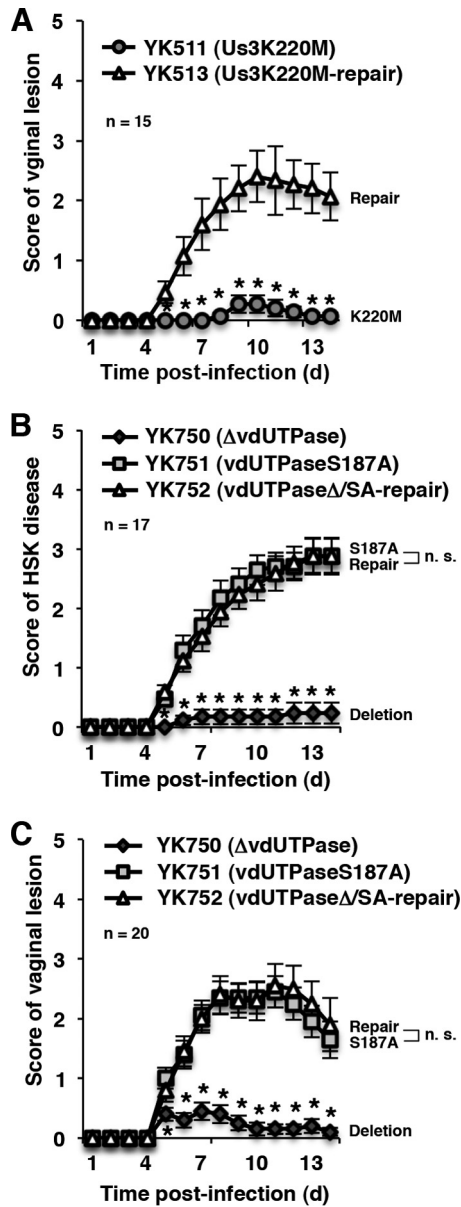


**FIG 2** Effect of mutations in vdUTPase or the kinase-dead mutation in Us3 on viral virulence in the CNS of mice following intracranial inoculation. (A to C) Sixteen (A and B) or seventeen (C) 3-week-old female ICR mice were infected intracranially with YK750 ( $\Delta$ vdUTPase) (A), YK751 (vdUTPaseS187A) (A and C), YK752 (vdUTPase $\Delta$ /SA-repair) (A and C), YK511 (Us3K220M) (B), YK513 (Us3K220M-repair) (B), YK753 (vdUTPaseS187D) (C), or YK754 (vdUTPaseS187D-repair) (C) and monitored for 14 days. *P* represents the statistical significance value according to the log-rank test. n.s., not significant.

(vdUTPaseD97A) or YK760 (vdUTPaseD97A-repair) (Fig. 8B and C and Fig. 9B and C). These results supported our hypothesis that vdUTPase enzymatic activity specifically regulated viral virulence and replication in the CNS of mice following intracranial inoculation but was not required for viral pathogenicity and replication in the eyes and vaginas of mice following ocular and vaginal inoculation. Furthermore, these results suggested that a currently unidentified vdUTPase activity, unrelated to its enzymatic activity, was critical for viral pathogenicity and replication in peripheral sites of mice following peripheral inoculation.



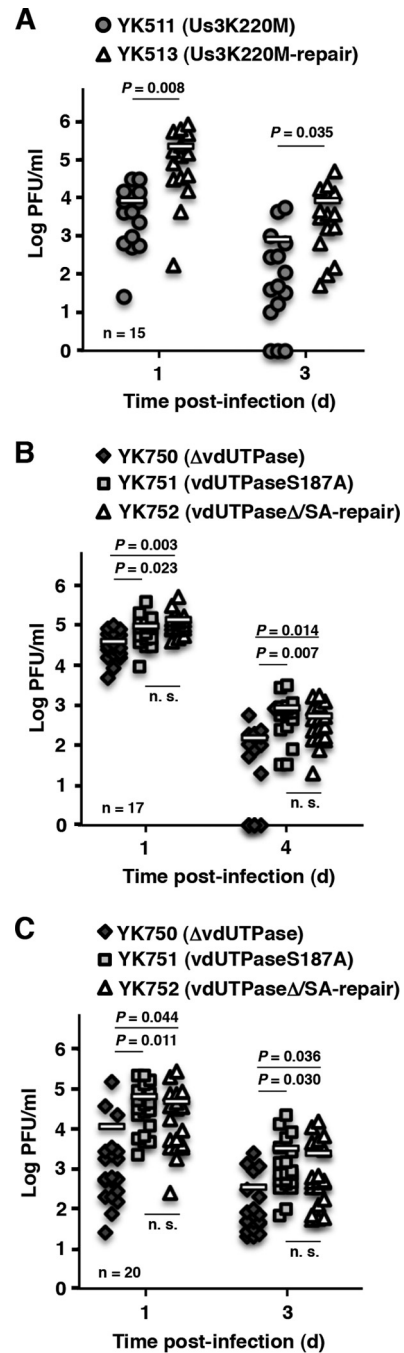
**FIG 3** Effect of mutations in vdUTPase or the kinase-dead mutation in Us3 on viral replication in the CNS of mice following intracranial inoculation. (A to C) Fifteen (A and B) or twenty-five (C) 3-week-old female mice were infected intracranially with YK750 ( $\Delta$ vdUTPase) (A), YK751 (vdUTPaseS187A) (A and C), YK752 (vdUTPase $\Delta$ /SA-repair) (A and C), YK511 (Us3K220M) (B), YK513 (Us3K220M-repair) (B), YK753 (vdUTPaseS187D) (C), or YK754 (vdUTPaseS187D-repair) (C). At 3 days postinfection, the brains of infected mice were harvested, and virus titers were assayed. Each data point is the virus titer in the brain of one mouse. The horizontal bars indicate the mean for each group. *P* represents the statistical significance value according to the two-tailed Student *t* test. n.s., not significant.



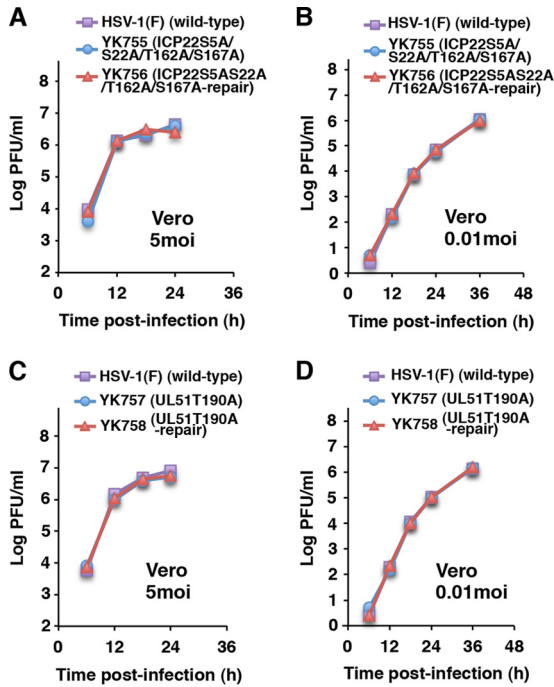
**FIG 4** Effect of mutations in vdUTPase or the kinase-dead mutation in Us3 on viral pathogenicity in peripheral sites of mice following peripheral inoculation. (A) Fifteen 5-week-old female ICR mice pretreated with Depo-M were infected vaginally with YK511 (Us3K220M) or YK513 (Us3K220M-repair) and scored for vaginal disease every day for 14 days. Each data point is the mean  $\pm$  the standard error of the scores. (B) Seventeen 5-week-old female ICR mice were ocularly infected with YK750 ( $\Delta$ vdUTPase), YK751 (vdUTPaseS187A), or YK752 (vdUTPase $\Delta$ /SA-repair) and scored for HSK every day for 14 days. Each data point is the mean  $\pm$  the standard error of the scores. (C) Twenty 5-week-old female ICR mice pretreated with Depo-M were infected vaginally with YK750 ( $\Delta$ vdUTPase), YK751 (vdUTPaseS187A), or YK752 (vdUTPase $\Delta$ /SA-repair). The experiments were performed as described for panel A. Each data point is the mean  $\pm$  the standard error of the scores. Asterisks represent the statistical significance value (\*,  $P < 0.05$ ) according to the two-tailed Student  $t$  test. n.s., not significant.

## DISCUSSION

We previously reported that vdUTPase Ser-187 is a physiological Us3 phosphorylation site in infected cells and that this phosphorylation promoted HSV-1 replication in a manner dependent on

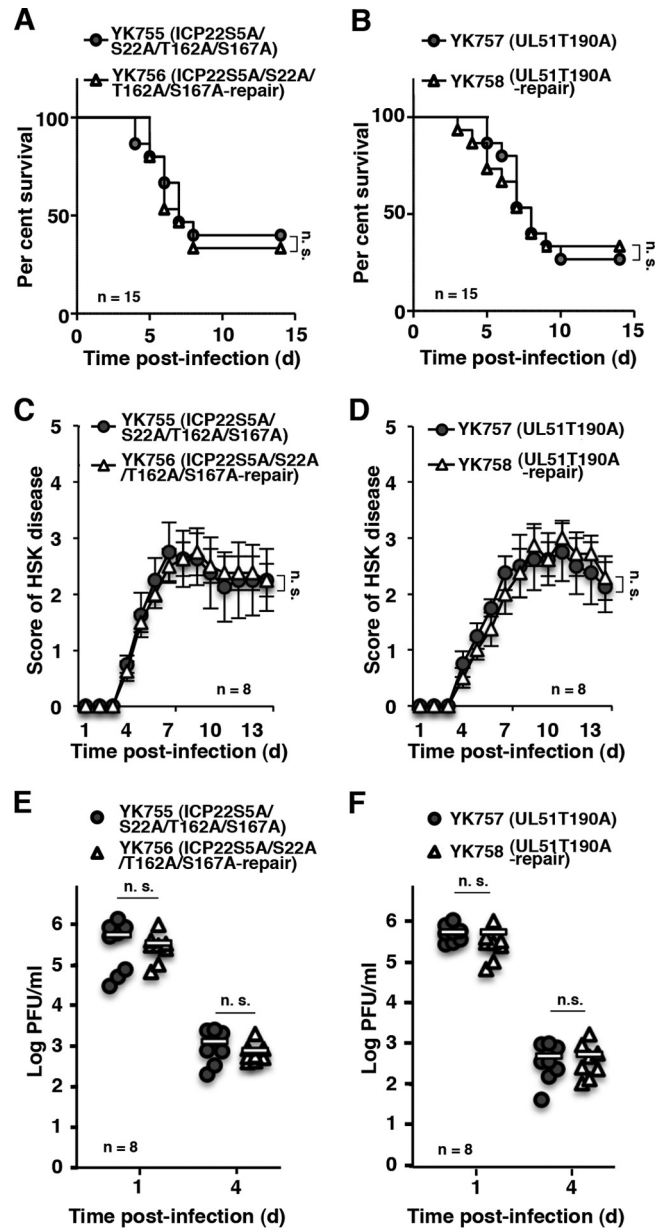


**FIG 5** Effect of mutations in vdUTPase or the kinase-dead mutation in Us3 on viral replication in peripheral sites of mice following peripheral inoculation. (A) The vaginal secretions of infected mice at 1 and 3 days postinfection in the experiment described in Fig. 4A were harvested, and virus titers were assayed. Each data point is the virus titer in the vaginal secretions of one mouse. The horizontal bars indicate the mean for each group. (B) The tear films of infected mice at 1 and 4 days postinfection in the experiment described in Fig. 4B were harvested, and virus titers were assayed. Each data point is the virus titer in the tear film of one mouse. The horizontal bars indicate the mean for each group. (C) The vaginal secretions of infected mice at 1 and 3 days postinfection in the experiment described in Fig. 4C were harvested, and virus titers were assayed. Each data point is the virus titer in the vaginal secretions of one mouse. The horizontal bars indicate the mean for each group.  $P$  represents the statistical significance value according to the two-tailed Student  $t$  test. n.s., not significant.



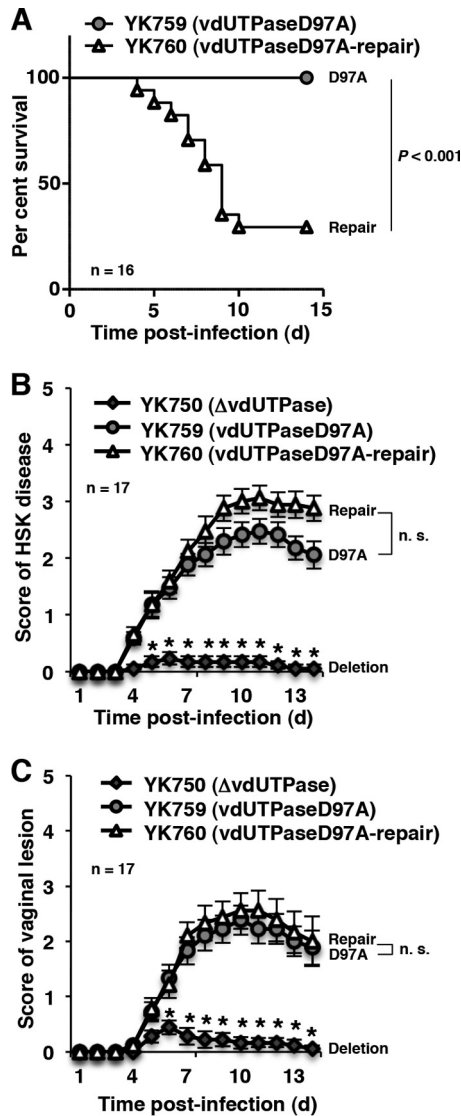
**FIG 6** Effect of phosphorylation of other viral proteins on viral replication in Vero cells. (A to D) Vero cells were infected at an MOI of 5 (left panels) or 0.01 (right panels) with wild-type HSV-1(F), YK755 (ICP22S5A/S22A/T162A/S167A), and YK756 (ICP22S5A/S22A/T162A/S167A-repair) (A and B) or wild-type HSV-1(F), YK757 (UL51T190A), and YK758 (UL51T190A-repair) (C and D). The total virus from the cell culture supernatants and infected cells was harvested at the indicated times and assayed on Vero cells. The data are representative of three independent experiments.

cell types and MOIs by regulating the optimal enzymatic activity of vdUTPase (17). However, the significance of Us3 phosphorylation of vdUTPase Ser-187 in viral replication and pathogenicity *in vivo* was uncertain. In the present study, we have shown that the vdUTPase S187A mutation significantly reduced mortality and viral replication in the brains of mice following intracranial inoculation and that the phosphomimetic substitution at vdUTPase Ser-187 in part restored the wild-type mortality and viral replication. In addition, the Us3 kinase-dead and vdUTPase enzyme-inactive mutations showed the phenotypes similar to the vdUTPase S187A mutation in mice following intracranial inoculation. Collectively, these observations suggested that the regulation of vdUTPase activity by Us3 phosphorylation of vdUTPase Ser-187 was critical for viral replication and virulence in the CNS of mice following intracranial inoculation. This is the first identification of a Us3 substrate involved in Us3 pathogenicity in the CNS of mice following intracranial inoculation. The more striking feature in the present study was that the phosphorylation, as well as the enzymatic activity, of vdUTPase played no role in viral replication and pathogenicity in the eyes and vaginas of mice following ocular and vaginal inoculation, respectively. To our knowledge, HSV proteins or their domains critical for viral replication and virulence in the CNS of mice following intracranial inoculation are, in almost all cases, also involved in pathogenicity in peripheral sites, such as eyes and vaginas, following peripheral (ocular and vaginal) inoculation (7, 10, 11, 15, 27, 41, 44–59). Therefore, this phosphorylation reaction, which promoted the vdUTPase activity, is a unique indication of a specific mechanism involved in HSV-1 replication and virulence in



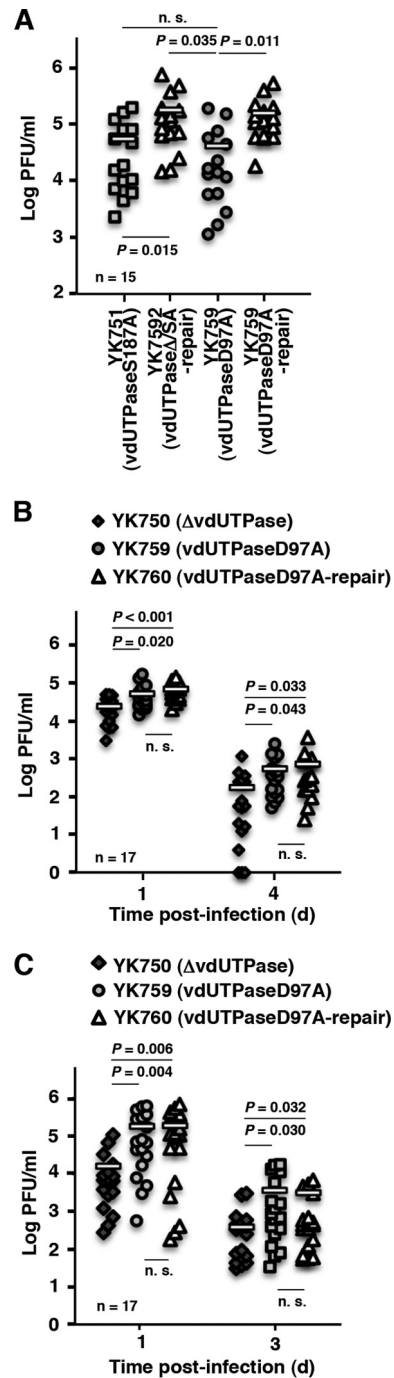
**FIG 7** Effect of phosphorylation of other viral proteins on viral pathogenicity and replication in mice. (A and B) Fifteen 3-week-old female ICR mice were infected intracranially with YK755 (ICP22S5A/S22A/T162A/S167A) and YK756 (ICP22S5A/S22A/T162A/S167A-repair) (A) or YK757 (UL51T190A) and YK758 (UL51T190A-repair) (B), and the survival of infected mice was monitored for 14 days postinfection. (C and D) Eight 5-week-old female ICR mice were ocularly infected and scored for severity of HSK every day for 14 days postinfection with YK755 (ICP22S5A/S22A/T162A/S167A) and YK756 (ICP22S5A/S22A/T162A/S167A-repair) (C) or with YK757 (UL51T190A) and YK758 (UL51T190A-repair) (D). Each data point is the mean  $\pm$  the standard error of the observations. (E and F) The tear films of infected mice at 1 and 4 days postinfection in the experiments described in panels C and D, respectively, were harvested, and virus titers were assayed. Each data point is the virus titer in the tear film of one mouse. The horizontal bars indicate the mean for each group. The statistical significance values were analyzed according to the log-rank test (A and B) or the two-tailed Student *t* test (C to F). n.s., not significant.





**FIG 8** Effect of the enzymatically inactive mutation in vdUTPase on viral pathogenicity in mice. (A) Sixteen 3-week-old female ICR mice were infected intracranially with YK759 (vdUTPaseD97A) and YK760 (vdUTPaseD97A-repair) and monitored for 14 days. (B) Seventeen 5-week-old female ICR mice were ocularly infected with YK750 ( $\Delta$ vdUTPase), YK759 (vdUTPaseD97A), and YK760 (vdUTPaseD97A-repair) and scored for HSK as described for Fig. 4B. Each data point is the mean  $\pm$  the standard error of the scores. (C) Seventeen 5-week-old female ICR mice pretreated with Depo-M were infected with YK750 ( $\Delta$ vdUTPase), YK759 (vdUTPaseD97A), and YK760 (vdUTPaseD97A-repair), and the score of vaginal disease in mice was analyzed as described for Fig. 4C. Each data point is the mean  $\pm$  the standard error of the scores.  $P$  represents the statistical significance value according to the log-rank test (A) or the two-tailed Student  $t$  test (B and C). n.s., not significant.

the CNS of mice following intracranial inoculation. We should note that HSV thymidine kinases (TKs) might behave similarly in the phosphorylation and the enzymatic activity of vdUTPase. However, roles of the TKs in viral replication at the periphery have been controversial, although the importance of the TKs in viral replication and virulence in the CNS was well established (48). Thus, several groups reported that the null mutations in the TKs had no obvious effect on viral replication in eyes of mice or guinea pigs following ocular inoculation (46, 60, 61), whereas other



**FIG 9** Effect of the enzymatically inactive mutation in vdUTPase on viral replication in mice. (A) Fifteen 3-week-old female mice were infected intracranially with YK751 (vdUTPaseS187A), YK752 (vdUTPase $\Delta$ /SA-repair), YK759 (vdUTPaseD97A), or YK760 (vdUTPaseD97A-repair). At 3 days postinfection, the brains of infected mice were harvested, and virus titers were assayed. Each data point is the virus titer in the brain of one mouse. The horizontal bars indicate the mean for each group. (B) The tear films of infected mice at 1 and 4 days postinfection in the experiment described in Fig. 8B were harvested, and virus titers were assayed. Each data point is the virus titer in the tear film of one mouse. The horizontal bars indicate the mean for each group. (C) The vaginal secretions of infected mice at 1 and 3 days postinfection in the experiment described in Fig. 8C were harvested, and virus titers were assayed. Each data point is the virus titer in the vaginal secretions of one mouse. The horizontal bars indicate the mean for each group.  $P$  represents the statistical significance value according to the two-tailed Student  $t$  test. n.s., not significant.



groups demonstrated that these mutations significantly impaired viral replication in eyes, ears, or vaginas of mice following ocular, ear, or vaginal inoculation, respectively (49, 62–64).

Our observations raised the interesting question of why this phosphorylation was needed for infection of the CNS of mice but not for infection of peripheral sites. Many viruses, including poxviruses, adenoviruses, D-type retroviruses, and African swine fever virus (ASFV), encode dUTPases in addition to herpesviruses (29, 65–67). In agreement with our recent report that HSV-1 dUTPase activity was required for efficient viral replication in a manner dependent on cell types and MOIs, replication of recombinant ASFV and D-type retroviruses (e.g., equine infectious anemia virus, feline immunodeficiency virus, and caprine arthritis encephalitis virus) carrying mutations in each of the viral dUTPases was severely affected in specific cell types, especially in nondividing cells, whereas replication in actively dividing cells was only minimally decreased (68–72). Based on these observations, it has long been thought, but not proven, that viruses encode a vdUTPase to compensate for low cellular dUTPase activity if that is the situation in their host cells, e.g., in resting and differentiated cells, such as neurons and macrophages, where cellular dUTPase activity has been suggested to be low (70, 73). Therefore, one possible explanation for the unique mechanism mediated by Us3 phosphorylation of vdUTPase for the CNS-specific viral replication and virulence may be that the vdUTPase enzymatic activity was upregulated by Us3 phosphorylation of vdUTPase Ser-187 to compensate for the low cellular endogenous dUTPase activity in the CNS of mice for efficient viral replication and virulence. Further experiments to directly prove this hypothesis, e.g., data that overexpression of cellular dUTPase compensates for the reduction in replication and/or pathogenesis of viruses carrying mutations in vdUTPase in cell cultures and/or *in vivo*, will be needed to clarify this subject and are in progress in this laboratory.

Us3 phosphorylation of vdUTPase Ser-187 was not essential for vdUTPase activity but appeared to optimize vdUTPase activity in infected cells, based on our recent report that the S187A mutation in vdUTPase only reduced vdUTPase enzymatic activity in infected cells to 68% of that in wild-type virus-infected cells (17). In contrast, the data presented here indicated that this phosphorylation was critical for efficient viral replication and virulence in the CNS of mice following intracranial inoculation. These results suggested that vdUTPase enzymatic activity in infected cells was tightly regulated by Us3 phosphorylation of vdUTPase Ser-187, and this strict regulation was critical for efficient viral replication and virulence in the CNS of mice. dUTPase hydrolysis of dUTP to dUMP and pyrophosphate may have a dual effect on HSV-1 replication in the CNS: the prevention of the misincorporation of dUTP into replicating viral genomes and the promotion of dTTP synthesis (29, 74). In the CNS, composed of mostly nondividing cells, the deoxynucleoside triphosphate (dNTP) pools are limited since cellular genome replication does not take place, and therefore vdUTPase activity might unbalance the dNTP pools. It is well established that appropriately balanced dNTP pools are critical for the maintenance of cell homeostasis (75). It has been reported that an imbalance in dNTP pools can result in cell death, dysfunction of mitochondria, mutation, recombination, enhanced sensitivity to mutagens, and chromosome breakage, exchange, or loss. Thus, we suggest that the Us3 phosphorylation site in vdUTPase may have evolved to tightly regulate vdUTPase enzymatic activity in its host cells, such as the CNS.

We have shown that the vdUTPase null mutation reduced viral replication and pathogenicity in mice, both in the CNS following intracranial inoculation and in peripheral sites following inoculation at these sites. In contrast, S187A and D97A mutations in vdUTPase, which reduced or abolished vdUTPase enzymatic activity, both reduced viral replication and pathogenesis in the CNS of mice following intracranial inoculation, but not in the eyes and vaginas of mice following inoculation in these sites. These results indicated that a currently unidentified activity of the vdUTPase protein, which is unrelated to its enzymatic activity, was critical for viral replication and pathogenesis at peripheral sites of mice. Two recent reports are in agreement with this conclusion. It has been reported that murine gammaherpesvirus-68 (MHV-68) and Kaposi's sarcoma-associated herpesvirus (KSHV) vdUTPases inhibited type I interferon signaling by inducing degradation of the type I interferon receptor protein (76) and cell surface expression of NKp44L, a ligand for natural killer cell activating receptor NKp44 (77), respectively, independent of their enzymatic activity (76, 77). In addition, Epstein-Barr virus (EBV) vdUTPase has been reported to function as a pathogen-associated molecular pattern, to be recognized by Toll-like receptor 2, and to induce expression of several pro- and anti-inflammatory cytokines when unstimulated resting human peripheral blood mononuclear cells and human monocyte-derived macrophages were cultured with purified EBV vdUTPase proteins (78, 79). The biological significance of the immune modulations mediated by KSHV and EBV vdUTPases in the context of viral infection remains to be determined, and this KSHV vdUTPase function has been shown to not be conserved in homologs in other herpesviruses, including HSV-1 and EBV (77). However, it is of interest to investigate whether an HSV-1 vdUTPase activity unrelated to its enzymatic activity is involved in the immune modulations observed with MHV-68 and EBV vdUTPases. Furthermore, HSV-1 vdUTPase is a major virion component (17, 80), indicating that it has a role in the viral replication cycle, perhaps as a structural protein in virion maturation. Further studies to clarify the activity of vdUTPase unrelated to its enzymatic activity are needed and are in progress in our laboratories.

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