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Human Thymidine Kinase Can Functionally Replace Herpes Simplex Virus Type 1 Thymidine Kinase for Viral Replication in Mouse Sensory Ganglia and Reactivation from Latency upon Explant

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Herpes simplex virus type 1 thymidine kinase exhibits a strikingly broad substrate specificity. It is capable of phosphorylating deoxythymidine and deoxyuridine as does human thymidine kinase, deoxycytidine as does human deoxycytidine kinase, the cytosolic kinase whose amino acid sequence it most closely resembles, and thymidylate as does human thymidylate kinase. Following peripheral inoculation of mice, viral thymidine kinase is ordinarily required for viral replication in ganglia and for reactivation from latency following ganglionic explant. To determine which activity of the viral kinase is important for replication and reactivation in mouse ganglia, recombinant viruses lacking viral thymidine kinase but expressing individual human kinases were constructed. Each recombinant virus expressed the appropriate kinase activity with early kinetics following infection of cultured cells. The virus expressing human thymidine kinase exhibited thymidine phosphorylation activity equivalent to ~5% of that of wild-type virus in a quantitative plaque autoradiography assay. Nevertheless, it was competent for ganglionic replication and reactivation following corneal inoculation of mice. The virus expressing human thymidylate kinase was partially competent for these activities despite failing to express detectable thymidine kinase activity. The virus expressing human deoxycytidine kinase failed to replicate acutely in neurons or to reactivate from latency. Therefore, it appears that low levels of thymidine phosphorylation suffice to fulfill the role of the viral enzyme in ganglia and that this role can be partially fulfilled by thymidylate kinase activity alone.

Viruses can provide examples of the evolution of proteins that serve one function into ones that serve other functions. The herpes simplex virus type 1 (HSV-1)-encoded thymidine kinase (TK) appears to be such a protein. HSV-1 TK is a multifunctional enzyme that possesses kinase activities normally performed by three separate enzymes. It phosphorylates deoxythymidine (dT) and deoxyuridine (dU) as does human TK (hTK), deoxycytidine (dC) as does human deoxycytidine kinase (hdCK), and thymidylate (dTMP) as does human TMP kinase (hTMPK) (6–8, 30, 31). There are no recognizable sequence similarities between HSV-1 TK and hTK (3, 4, 23). Rather, sequence alignments have detected similarities between herpesvirus TKs and hdCK (23) and to a lesser extent cellular TMPK and other nucleoside monophosphate kinases (NMPKs) (45). Interestingly, hdCK can phosphorylate deoxyadenosine and deoxyguanosine (1, 16, 18, 47), while HSV-1 TK can phosphorylate several purine analogs (13, 34). Although HSV-1 TK shares rather limited sequence homology with enzymes of the NMPK family, its structure contains a parallel five-stranded β sheet and a P loop characteristic of NMPKs (57). Such similarities suggest that herpesvirus TKs may have evolved from cellular dCK or from a cellular NMPK. In addition to the activities shared with cytosolic kinases, HSV-1 TK is also able to phosphorylate nucleoside analogs such as the thymidine analog bromovinyldeoxyuridine (BVdU) and the guanine derivative acyclovir (13, 34). The loss of viral TK activity is a common mechanism through which resistance to these drugs occurs.

The role of TK in HSV pathogenesis in animal models has drawn considerable attention in part because although it is not essential for viral replication in certain tissues, it is necessary for crucial events in sensory ganglia (14, 19, 29, 53). HSV infection of mammalian hosts involves both productive infection and latency. Following productive infection in peripheral tissues such as the cornea, the virus gains access to nerve terminals and, after an acute phase of productive replication in sensory ganglia, establishes and maintains a latent infection primarily, if not exclusively, in neurons. During latency, the productive cycle of viral gene expression is severely repressed and infectious virus is not detected, yet the latent virus can reactivate to cause recurrent disease (46, 56). In mice, both TK-competent and TK-negative (TK^-) HSV replicate equally well in the eye after corneal inoculation, but there is little or no acute virus replication or reactivation of virus from the latent state in ganglia infected with TK^- HSV (14, 19, 29, 53).

Because of the unusual properties of HSV-1 TK, we wished to determine whether a heterologous kinase with more limited substrate specificity could fulfill the role of the viral enzyme in virus replication and reactivation in ganglia. Therefore, recombinant viruses lacking HSV-1 TK, but expressing hTK, hdCK, or hTMPK, were constructed and tested for the ability to grow and reactivate in mouse sensory ganglia following corneal inoculation.

MATERIALS AND METHODS

Cells and viruses. Vero and TK^- human osteosarcoma (143) cells were propagated and maintained as described previously (55). Wild-type HSV-1 strain KOS, HSV-1 mutants *tk*LTRZ1 (17), 615.9 (25), and KG111 (13, 26), and a series of linker scanning (LS) *tk* promoter mutants (LS-95/-85, LS-111/-101//-56/-

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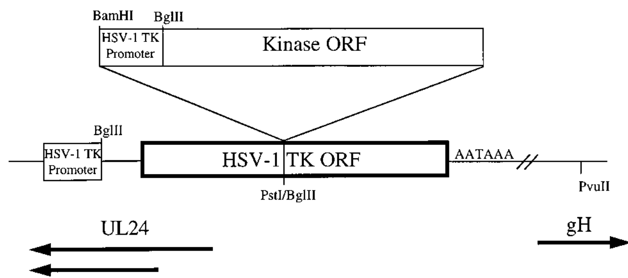

FIG. 1. Construction of plasmids carrying a copy of the HSV-1 *tk* promoter and a human kinase gene within the viral *tk* coding region. The top line shows that each open reading frame (ORF) encoding the human kinases was inserted downstream of a copy of the HSV-1 *tk* promoter (a *Bam*HI-*Bgl*II fragment containing nt −105 to +53 relative to the *tk* mRNA start site). The next line shows the relative location (*Pst*I site, nt +801) in the HSV-1 *tk* at which the copy of the viral *tk* promoter and the human kinase ORF were inserted. The bottom line shows the transcriptional start sites and the orientations of *UL24* and *gH* transcripts.

46, and LS-29/-18) (2, 13, 15, 26) used in this study were grown and titrated as described previously (12).

Plasmids. p101086.7 (kindly provided by D. Yager) contains a ~1.8-kb *Bgl*II-*Pvu*II fragment (from +53 relative to the *tk* mRNA start site [26] to within the *gH* [*UL22*] gene) fragment derived from wild-type HSV-1 strain KOS cloned into pBluescript (Stratagene). This plasmid, which was constructed so that the *Bgl*II site was lost, was linearized with *Pst*I (which cuts at nucleotide [nt] +801 relative to the *tk* mRNA start site and is outside *UL*24 [Fig. 1]), recessed ends were made blunt with T4 DNA polymerase, *Bgl*II linkers were added by linker tailing, and the fragment was recircularized to produce 101086.7.BglII. A 158-bp *Bam*HI-*Bgl*II fragment containing the *tk* promoter (nt −105 to +53) isolated from pLS/ts-115/-105 (15) was then inserted at the *Bgl*II site in 101086.7.BglII, leaving a single *Bgl*II site intact downstream of the promoter. The LS-115/-105 mutant exhibits wild-type promoter activity (15, 41). A plasmid with the *tk* promoter in the proper orientation was designated 101086.7.Pro. p1010.hTK was constructed by inserting a 1.45-kb *Bam*HI-*Bam*HI fragment isolated from pTK11 (4) (kindly provided by P. L. Deininger) into the *Bgl*II site in 101086.7.Pro. p1010.hdCK was constructed by isolating a 1.17-kb *Xho*II-*Xho*II fragment from pCD1 (11) (kindly provided by B. S. Mitchell), blunt ending with T4 polymerase, and adding *Bgl*II linkers. Following digestion with *Bgl*II, the fragment was inserted into the *Bgl*II site in 101086.7.Pro. p1010.hTMPK was constructed by digesting p561 (52) (kindly provided by R. A. Sclafani) with *Eco*RI, blunt ending with Klenow fragment, and then adding *Bgl*II linkers. The DNA was then digested with *Bgl*II which also cut at a site in the 3′ end of the hTMPK gene to liberate a 860-bp fragment which was then inserted into the *Bgl*II site in p101086.7.Pro. DNA sequencing verified that each insert was in the proper orientation and had the expected junctions.

Virus construction. Plasmid DNA was linearized with *Sal*I, which cleaves in vector sequences, and cotransfected as described previously (10) with either infectious KOS or *tk*LTRZ1 DNA. Recombinant viruses expressing hTK were obtained by transfecting plasmid p1010.hTK with infectious *tk*LTRZ1 DNA and screening progeny virus in the presence of 300 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. Recombinant hdCK and hTMPK viruses were obtained by transfecting the corresponding plasmids with infectious KOS DNA and then determining progeny virus titers in the presence of 15 mM BVdU. White (hTK) or BVdU-resistant (hdCK and hTMPK) plaques were picked; viral DNA was prepared as described previously (15) and PCR amplified with primers TK9 (25) and TK8 (CCGAACCCCGCGTTTATGAACA; complementary to *tk* nt +1326 to +1347). Isolates of recombinant virus containing the hTK gene generated a ~1.4-kb PCR product which was easily distinguished from a 3.4-kb product from the parental strain *tk*LTRZ1. Isolates of the recombinant virus containing the hdCK and hTMPK genes generated longer PCR products, ~1.2 and 0.8 kb, respectively, than the 21-bp product generated from the parental strain KOS. Two independent isolates (from independent transfections) were obtained for each recombinant virus. Each isolate was plaque purified two more times and then used to prepare high-titer stocks. The purity of virus in high-titer stocks was confirmed by Southern blot hybridization using probes from the genes encoding viral TK (~500-bp *Bgl*II-*Sac*I fragment), hTK (~790-bp *Mlu*I-*Hin*dIII fragment), hdCK (~530-bp *Pst*I-*Hin*dIII fragment), and hTMPK (~260-bp *Pvu*II-*Pst*I fragment).

Enzyme assays. A subconfluent monolayer of 10^6 143 cells, which lack cytosolic hTK, was infected with the indicated virus at a multiplicity of infection of 10. Cells were harvested at various times postinoculation (p.i.) and lysed by sonication in buffer containing 100 mM Tris-Cl (pH 7.5), 10% glycerol, 1 mM dithiothreitol, and 40 μM ATP-Mg^{2+}. The samples were microcentrifuged at 4°C (14,000 × *g*), and kinase assays were performed with 5 to 10 μl of supernatant. Kinase reaction mixtures contained 0.1 M Tris-Cl (pH 7.5), 2 mM dithiothreitol, 6 mM $MgCl_2$, 6 mM ATP, 7 mM NaF, 1 U of creatine phosphokinase, 6 mM creatine phosphate, and 0.1 mM radiolabeled substrate (150 mCi of [5-^{3}H]dC, 150 mCi of [*methyl*-^{3}H]dT, or 70 mCi of [2-^{14}C]TMP per mmol; Moravek) in a total volume of 80 μl. Reactions were allowed to proceed at 37°C for 60 to 90 min; 50 μl of each reaction mixture was applied to a DE-81 anion-exchange disc. For dC and dT kinase assays, the discs were washed three times in 1 mM ammonium formate and once in 95% ethanol. For TMPK assays, reaction mixtures were applied to DE-81 discs that had been presoaked in 10 mM TMP and dried. The discs were washed twice in 0.1 M formic acid, once in 1 mM TMP, and once in 95% ethanol. The discs were dried, and radioactivities were determined by scintillation spectrometry. Enzyme activities were normalized to protein concentrations, which were determined by Bradford assay, and values from mock-infected cells were subtracted from values from infected cells. The dC and dT kinase activities in extracts of mock-infected cells were <1% of that of KOS-infected cells, while the dTMPK activity in extracts of mock-infected cells was 30% of that of KOS-infected cells. Kinase activities were expressed as a percentage of wild-type KOS activity after subtraction of mock values. Limits of detection were determined by calculating the lowest percentage of KOS activity at which kinase activity was detectably greater than that of mock-infected cells.

Plaque autoradiography. The procedure of Martin et al. (40) was modified to permit quantification of TK activity in situ, using thymidine labeled to high specific activity with ^{3}H and storage phosphor technology. Briefly, ~120 PFU of each virus was inoculated onto a 60-mm-diameter petri dish seeded the day before with 2×10^5 143 cells, and the infected cells were overlaid with 0.75% methylcellulose-containing medium. After 5 days at 34°C, the overlay was removed and the monolayer was incubated with 2.6 μCi of [^{3}H]thymidine (*methyl*-^{3}H, 64.5 Ci/mmol; Moravek) per ml in medium for 8 h at 34°C. The cells were then stained with 2% crystal violet in 10% ethanol, washed, and air dried. Circumferential rims were removed, and the plates were exposed for 6 days to a tritium plate from a phosphorimager (Fuji), which was then scanned to obtain images and quantitative data. To measure the relative amount of TK activity, the radioactivity from 10 representative plaques from each dish was quantified according to the manufacturer's protocol, background radioactivity from a section of the image similar in size to plaques was subtracted, and the data were normalized to those obtained with strain KOS. To calibrate the assay, we used mutant KG111, which expresses 10% of wild-type levels of TK polypeptide, TK activity in infected cells, and TK activity in infected cell extracts (13, 26), LS-95/-85, LS-111/-101//-56/-46, and LS-29/-18, which express different amounts of *tk* mRNA and correspondingly ~5, 2, and 0.5% of wild-type levels of active TK polypeptide (2, 13, 15, 26), and mutant 615.9, which expresses ~1% of wild-type levels of TK polypeptide (25) and TK activity in this assay (24).

Assays of acute and latent infections in mice. Eight-week-old Hsd:ICR mice (Harlan Sprague Dawley) were inoculated on the cornea with wild-type virus or recombinant viruses as described previously (13, 38) at a dose of 2×10^6 PFU/eye. Virus titers at the site of inoculation and trigeminal ganglia were assayed by swabbing eyes 1 day p.i. and by excising and homogenizing ganglia 3 days p.i. Thirty days after inoculation, trigeminal ganglia were excised and tested for the presence of reactivatable virus by a dissociation method as previously described (38) except that cultures were screened for 10 days following explant and 4 days following replating.

RESULTS

Construction of recombinant viruses carrying the genes for human kinases under the control of the HSV-1 *tk* promoter. To construct viruses expressing individual human kinases, we first constructed plasmids in which the open reading frame of the human kinase was inserted downstream of a second copy of the viral *tk* promoter at a site located within the *tk* coding region (Fig. 1). Following recombination with viral DNA, this would generate recombinant viruses that express human kinase activities under the control of this second *tk* promoter while simultaneously inactivating the HSV-1 *tk* gene. The site of insertion within the *tk* gene was far from the *UL24* gene (Fig. 1), which overlaps the 5′ end of the *tk* gene and is important for productive ganglionic infection in mice (27, 28). Sequence analyses of these plasmids verified that each insert was in the proper orientation and had the expected junctions.

The recombinant virus encoding hTK was constructed by cotransfection of the respective plasmid with infectious DNA from *tk*LTRZ1, which forms blue plaques in the presence of X-Gal due to a *lacZ* insertion in the *tk* gene (17). Recombination was therefore expected to replace the *lacZ* gene, thus giving rise to white plaques in the presence of X-Gal. Recombinant viruses encoding hdCK and hTMPK were constructed by cotransfection of the relevant plasmid with infectious DNA

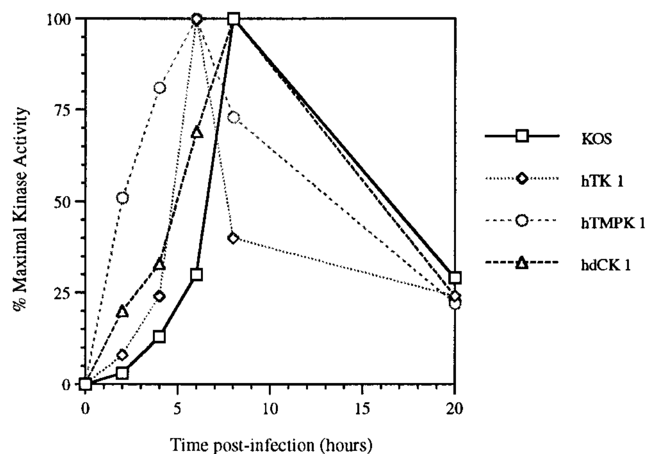

FIG. 2. Time courses of kinase activities in cells infected with recombinant viruses. Extracts of infected cells were prepared at the indicated times p.i., and enzyme assays were performed as described in Materials and Methods. Values from mock-infected cells were subtracted, and the percentage of maximal activity was calculated for each time point. The data shown are the average of two separate sets of assays, each done in triplicate. The standard deviations for each time point were small relative to the value determined. Similar results were obtained in a second time course experiment, which was also assayed twice, each time in triplicate. The maximal activity (100%) occurred at 8 h p.i. for KOS and hdCK1 and 6 h p.i. for hTK1 and hTMPK1.

from wild-type HSV-1 strain KOS. Recombination was expected to give rise to virus deficient in viral TK that would be resistant to drugs such as BVdU. White or BVdU-resistant plaques were picked, viral DNA was prepared, and PCR assays were performed to confirm the sizes of inserts. Isolates containing the desired inserts were plaque purified and confirmed by PCR assays two more times and then used to prepare high-titer stocks. Two independent isolates were obtained for each recombinant virus to ensure that any phenotypes were due to the engineered insertion. The identity and purity of high-titer stocks were confirmed by Southern blot hybridization using probes from the genes encoding HSV-1 TK, hTK, hdCK, and hTMPK (data not shown). The viruses encoding hTK were designated as hTK1 and hTK2, those encoding hdCK were designated hdCK1 and hdCK2, and those encoding hTMPK were designated hTMPK1 and hTMPK2. All of the recombinant viruses replicated to high titers in cell culture and formed plaques that were the same size as those of KOS.

Enzymatic activities of recombinant kinase viruses. To determine whether the recombinant viruses expressed active forms of the expected kinases, enzyme assays were performed on extracts of 143 TK$^-$ cells that had been infected with strain KOS, hTK1, hdCK1, or hTMPK1. All three recombinant viruses expressed the expected kinase activity with early kinetics similar to that of wild-type HSV-1 (Fig. 2). Viruses hTK1 and hTMP1 expressed activities that peaked at 6 h p.i., while KOS and hdCK1 expressed activities that peaked at 8 h p.i. The enzymes expressed by the recombinant viruses exhibited the expected substrate specificities (18, 21, 37, 43): extracts from cells infected with hTK1 phosphorylated dT, but not dTMP or dC, detectably; extracts of cells infected with hTMPK1 phosphorylated dTMP, but not dT or dC, detectably; and extracts of cells infected with hdCK1 phosphorylated dC, but not dT or dTMP, detectably above the activities observed in extracts of mock-infected cells (Table 1). Extracts from KOS-infected cells efficiently phosphorylated all three substrates. Relative to KOS, hTK1 and hTMPK1 viruses expressed 37% TK and 70% TMPK activity, respectively, in these assays. Recombinant hdCK1 virus exhibited dCK activity that was 7.2% of that of KOS. Although this value was low, it was well above the limits of detection. Moreover, previous studies have shown that a virus expressing similar levels of active viral TK polypeptide is fully competent for ganglionic replication and reactivation in mice (13, 26).

Quantitative plaque autoradiography. To assess the TK activities of the recombinants more authentically, we used an assay to quantify phosphorylation of thymidine in situ (40). We calibrated the assay by using a series of HSV mutants, each expressing different amounts of active viral TK polypeptide. To increase sensitivity, the assay was performed by using conditions such that mutant KG111, which expresses ~10% of wild-type levels of active TK polypeptide and is fully competent to reactivate from latency (13, 26), yielded a signal almost as intense as that of wild-type virus (Fig. 3). For KG111 and a series of mutants that express ~5, 2, and 1% of wild-type levels of active TK polypeptide (2, 13, 15, 26), the amounts of radioactivity per plaque decreased monotonically with the amount of TK expressed (Fig. 3). However, the plaque autoradiography assay did not detect activity in plaques of mutant LS-29/-18, which expresses ~0.5% active TK polypeptide relative to wild type (15). Thus, the limit of detection of this assay was between 0.5 and 1% of wild-type TK activity. In this assay, the amount of radioactivity per plaque in the recombinant virus expressing hTK was equivalent to that of a mutant, LS-95/-85, which expresses ~5% active TK polypeptide relative to KOS (15). The discrepancy between this value and the value obtained in the enzymatic assay (Table 1) is likely due to the enzymatic assay being optimized for hTK, not HSV-1 TK, and to the rapid decline in hTK activity following its peak value (Fig. 2). No phosphorylation of thymidine by hdCK and hTMPK was detected by this assay (Fig. 3), consistent with our enzymatic assays (Table 1) and those of others (18, 37, 43).

Reactivation of recombinant kinase viruses in a mouse model. We next performed animal studies to determine which recombinant viruses were capable of ganglionic replication and reactivation in mouse sensory ganglia following corneal inoculation. Recombinant viruses hTK1, hTK2, hTMPK1, hTMPK2, hdCK1, and hdCK2, wild-type strain KOS, and the KOS-derived *tk* insertion mutant *tk*LTRZ1 achieved similar titers in eye swabs 24 and 48 h after infection (Table 2). At 72 h p.i., some of the mutant viruses exhibited lower titers, as is sometimes observed with certain TK$^-$ mutants (14). However, the titers of recombinant viruses hTK1, hTK2, and hdCK2 were indistinguishable despite marked differences in their ganglionic phenotypes (see below).

The two independent isolates of recombinant virus expressing hTK replicated efficiently in ganglia, achieving titers similar to those of strain KOS 3 days p.i. (Table 3). These viruses also reactivated from latency efficiently. Thus, the recombinant vi-

TABLE 1. Enzyme activity

Virus	Enzyme activity (% of KOS activity)[a]		
	dTK	dCK	dTMPK
hTK1	37 ± 3	—[b]	—
hdCK1	—	7.2 ± 2.4	—
hTMPK1	—	—	70 ± 9

[a] Values are from peak time points (8 h p.i. for KOS and hdCK1 and 6 h p.i. for hTK1 and hTMPK1) and are means ± standard deviations of six to nine determinations.

[b] —, below limit of detection. The limits for detection of activity significantly greater than that of mock-infected cells were 0.5% of KOS for dTK activity, 1.5% of KOS for dCK activity, and 32% of KOS for dTMPK activity.

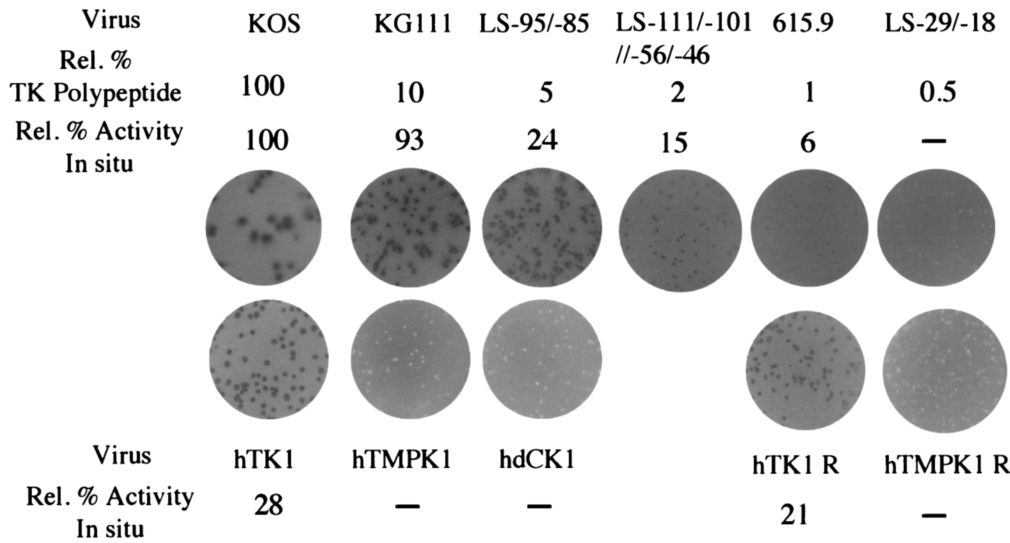

FIG. 3. Plaque autoradiography. TK⁻ human osteosarcoma (143) cells were inoculated with ~120 PFU of each virus, and plaque autoradiography was performed as described in Materials and Methods. The top line shows the approximate amounts of active TK polypeptide expressed by each mutant relative (Rel.) to wild-type strain KOS. The next line shows the average amount of radioactivity measured per plaque relative to KOS. (In each case, standard errors were negligible.) The images of the plates infected with the various viruses are provided in the next two lines. The bottom line indicates the amount of radioactivity measured per plaque relative to KOS for each of the plates above. A dash denotes no radioactivity detected above background. hTK1 R and hTMPK1 R were viruses reactivated from ganglia latently infected with these two viruses. Similar results were obtained with two independent isolates; images of only one isolate are shown.

rus expressing hTK was competent for ganglionic replication and reactivation, although its TK activity in situ was only ~5% of that of KOS.

The two independent isolates of virus expressing hTMPK exhibited a 1,000- to >10,000-fold reduction in ganglionic replication. Only 5 of 12 ganglia (42%), 2 from hTMPK1 and 3 from hTMPK2, were able to reactivate (Table 3). Thus, this virus was partially competent for ganglionic replication and reactivation. Viruses that reactivated from ganglia infected with hTK1, hTK2, hTMPK1, and hTMPK2 retained their TK phenotypes as determined by plaque autoradiography (Fig. 3 and data not shown) and genotypes as determined by Southern blot hybridization (data not shown). Thus, the ability of these viruses to reactivate was not due to reversion to or contamination with wild-type virus. In contrast to the hTK and hTMPK recombinants, both independent isolates of virus expressing hdCK failed to replicate acutely in ganglia or to reactivate from latency, similar to the KOS-derived TK⁻ mutant, *tk*LTRZ1 (Table 3).

DISCUSSION

HSV-1 TK possesses several kinase activities, including those found separately in hTK, hdCK, and hTMPK. To determine which kinase activity is important for replication and reactivation in mouse ganglia, viruses expressing these individual human kinases, whose substrate specificities are more limited than that of HSV-1 TK, were constructed. Our studies showed that the recombinant virus in which HSV-1 TK was replaced with hTK was competent for ganglionic replication and reactivation. Thus, there is no need to invoke a role for any of the unusual properties of HSV-1 TK in ganglionic replication and reactivation. As hTK lacks any known dCK or TMPK activity (Table 1 and references 20 and 21), these results strongly suggest that thymidine phosphorylation suffices to fulfill the role of HSV-1 TK in ganglia. This places on a firmer footing the widely accepted idea that HSV-1 TK functions to supply thymine nucleotide precursors for viral DNA replication. Presumably, other sources of phosphorylated dC are employed to support viral DNA replication in ganglia. The dependence on viral TK for ganglionic replication and reactivation may reflect the fact that hTK is strictly cell cycle controlled (22, 33, 42, 50) and is present in high levels only in rapidly dividing cells but would not be in nondividing neurons.

We have previously shown that there are 10- to 50-fold-fewer genomes and about 5-fold-fewer cells expressing latency-associated transcripts (LATs) in ganglia latently infected with TK⁻ mutants than in ganglia infected with wild-type virus (29, 32, 35, 36). The latter phenotype, at least, is due to a *tk* mutation (29). Numbers of viral genomes and cells expressing LATs are frequently taken as measures of the efficiency of establishment of latency. By these criteria, then, TK can affect the efficiency of this process. However, several studies argue convincingly that this decrease in efficiency of establishment cannot explain the requirement for TK for reactivation from latency. Several mutants are at least as defective as TK⁻ mutants for numbers of viral genomes and/or LAT-expressing

TABLE 2. Virus titers in eye swabs during acute infections of mice[a]

Virus	Titer (log mean[b] ± SE)		
	Day 1 p.i.	Day 2 p.i.	Day 3 p.i.
KOS	6.2 ± 0.2 (4)	4.6 ± 0.2 (4)	4.0 ± 0.2 (4)
hTK1	6.1 ± 0.1 (2)	3.7 ± 0.5 (2)	3.6 ± 0.4 (2)
hTK2	5.9 ± 0.1 (2)	4.6 ± 0.4 (2)	3.4 ± 0.6 (2)
hTMPK1	5.8 ± 0.1 (2)	3.2 ± 0.6 (2)	1.6 ± 1.6 (2)
hTMPK2	5.8 ± 0.2 (2)	3.8 ± 0 (2)	0.4 ± 0.4 (2)
hdCK1	4.8 ± 0.8 (2)	3.5 ± 0.6 (2)	0.7 ± 0 (2)
hdCK2	6.0 ± 0.3 (2)	3.3 ± 0.2 (2)	3.3 ± 0.2 (2)
*tk*LTRZ1	6.1 ± 0.1 (4)	3.7 ± 0.4 (4)	2.5 ± 0.5 (4)

[a] Mice were inoculated corneally with 2×10^6 PFU of the indicated viruses. Virus replication in the eye was assessed by titrating eye swabs at the time points indicated. The number of samples titrated for each group is shown in parentheses.

[b] Calculated by averaging the logs of the titers.

TABLE 3. Acute and latent infections in ganglia of mice[a]

Virus	Titer in ganglia (log mean[b] ± SE)	Ganglia reactivating/ total ganglia
KOS	5.2 ± 0.1 (4)	8/8
hTK1	5.4 ± 0.1 (2)	6/6
hTK2	3.1 ± 0.8 (2)	5/6
hTMPK1	1.0 ± 0 (2)	2/6
hTMPK2	2.2 ± 0.8 (2)	3/6
hdCK1	<1 (2)	0/6
hdCK2	<1 (2)	0/6
*tk*LTRZ1	<1 (4)	0/8

[a] Mice were inoculated corneally with 2×10^6 PFU of the indicated viruses. Three days postinfection, ganglia were harvested and titrated for virus; 30 days postinfection, ganglia were harvested for reactivation of latent virus by a dissociation assay. The number of samples titrated for each group is shown in parentheses.

[b] Calculated by averaging the logs of the titers.

cells in trigeminal ganglia, yet these mutants are qualitatively capable of reactivation (5, 27, 44, 48, 49, 51). Trigeminal ganglia from mice infected with low doses of wild-type virus contain even fewer numbers of viral genomes yet reactivate relatively efficiently (9). Reactivation from trigeminal ganglia containing high numbers of wild-type genomes is drastically inhibited by specific inhibitors of HSV TK (29, 39). Thus, ordinarily, viral TK is specifically required for reactivation and hTK can replace viral TK for this function.

Based on plaque autoradiography assays (Fig. 3) and assays of acute and latent infections in mice, hTK activity equivalent to only ~5% of wild-type viral TK activity was sufficient for ganglionic replication and reactivation similar to that of wild-typ virus. We have previously shown that ~10% viral TK activity is sufficient for reactivation from latency (13, 26). The present result extends this previous finding and indicates that, at least in mice, HSV-1 expresses much more TK activity than is required for its ganglionic functions.

The virus expressing hTMPK was partially competent for ganglionic replication and reactivation. One explanation for these results could be that hTMPK is capable of phosphorylating dT at low levels. Arguing against this interpretation are our failure to detect TK activity in two sensitive assays and previous studies of this enzyme (37). An alternative interpretation is that expression of hTMPK enables reactivation by increasing the phosphorylation of TMP from cellular sources. Potential sources of TMP include mitochondrial TK and the dCMP deaminase/thymidylate synthase pathway for conversion of dCMP to dTMP. However, the levels of these enzymes in neurons are not known. Tenser et al. (54) previously observed that supraphysiologial concentrations of thymidine but not uridine, dU, or dC could overcome the reactivation defects of TK^- viruses. Perhaps this led to synthesis of TMP from mitochondrial TK or residual cytosolic TK that could then be phosphorylated by cellular TMPK. Taken together, the results suggest that any change favoring increased TTP formation may enable ganglionic replication and reactivation.

We have recently deleted much of the *tk* gene from a clinical isolate yet the resulting mutant, GG*dl*tk, is partially competent to reactivate from mouse ganglia (24). Initial studies raise the possibility that this virus may contain alleles that compensate for the loss of TK during reactivation. In line with the ability of the virus expressing hTMPK to reactivate from latency, perhaps increased activities of other viral nucleotide-metabolizing enzymes (e.g., dUTPase and ribonucleotide reductase) partially fulfill the role of TK in GG*dl*tk.

One implication of the reactivation competence of the hTMPK viruses is that an HSV-1 mutant that was TK^- but $TMPK^+$ might be expected to reactivate despite lacking TK activity. It is possible that certain acyclovir-resistant isolates that have been reported to be TK^- could be mutants of this type.

Two independent isolates of the virus expressing hdCK did not replicate in ganglia or reactivate. However, dCK expression in hdCK1-infected cells was low, and it is possible that higher levels of dCK, which can lead to increased thymidine production via the dCMP deaminase/thymidylate synthase pathway, might have led to some restoration of replication and reactivation. If one could engineer a virus that expresses high levels of hdCK with appropriate kinetics, this question could be addressed. Nevertheless, low levels of hTK, which does not detectably phosphorylate dC (Table 1 and references 20 and 21), sufficed to replace HSV-1 TK. Although hdCK is the enzyme with which it shares the highest degree of sequence similarity (23), the ability of HSV-1 TK to phosphorylate dC does not appear to play an important role in the virus life cycle. It may be that the ability of HSV-1 TK to phosphorylate dC has no adaptive significance but rather is simply a vestige of its evolutionary origins.

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