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Expression of Extremely Low Levels of Thymidine Kinase from an Acyclovir-Resistant Herpes Simplex Virus Mutant Supports Reactivation from Latently Infected Mouse Trigeminal Ganglia▽

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A single-cytosine-deletion in the herpes simplex virus gene encoding thymidine kinase (TK) was previously found in an acyclovir-resistant clinical isolate. A laboratory strain engineered to carry this mutation did not generate sufficient TK activity for detection by plaque autoradiography, which detected 0.25% wild-type activity. However, a drug sensitivity assay suggested that extremely low levels of TK are generated by this virus. The virus was estimated to express 0.09% of wild-type TK activity via a ribosomal frameshift 24 nucleotides upstream of the mutation. Remarkably, this appeared to be sufficient active TK to support a low level of reactivation from latently infected mouse trigeminal ganglia.

Herpes simplex virus (HSV) thymidine kinase (TK) selectively activates a number of highly effective antiviral drugs, including acyclovir (ACV). However, pathogenic viruses that are resistant to ACV frequently arise in immunocompromised patients (3, 9). Many drug-resistant HSV isolates generate low levels of active TK, and it has been proposed that these viruses generate insufficient TK to activate ACV but enough to support pathogenesis in an immunocompromised patient (14, 19). The mutation that most commonly confers resistance is a single-guanine (G) insertion in a run of seven Gs ("G string") in *tk* (10, 11, 22, 23). Viruses with this mutation generate low levels of full-length active TK via a net +1 ribosomal frameshift that compensates for the added base (12, 18, 19, 26). Further studies revealed that the wild-type-length (G_7) G string appeared to support both +1 and −1 ribosomal frameshifting (15).

A drug-resistant virus shown to have a deletion of a cytosine (C) in a run of 5 Cs 24 nucleotides downstream of the G string was previously isolated from the oropharynx of an individual with an unknown history of immunosuppression (10). (Fig. 1). Given that the wild-type G string (G_7) was sufficient for −1 ribosomal frameshifting (15), we hypothesized that a −1 ribosomal frameshift on the G string could compensate for the deleted C downstream. In this scenario, the ribosome would shift the reading frame on the G string and translate non-wild-type amino acids between the G string and the deleted C. The wild-type reading frame would be restored due to the deleted C. Enzyme activity would be dependent on the efficiency of the −1 ribosomal frameshift on the wild-type G string, previously estimated to be 0.25% (15), in addition to the effect of the non-wild-type amino acids between the G string and the Cs.

Construction of recombinant virus TK1C5-1C. To permit comparison with previously reported viruses, the deleted-C mutation was engineered into the laboratory strain KOS (Fig. 1). Strain KOS was chosen because KOS mutants that lack TK activity are unable to reactivate from latently infected mouse trigeminal ganglia (2, 6, 12, 13, 15, 20). The C deletion was introduced into plasmid pAG5 (13), which contains the entire BamHI "P" fragment from KOS, by site-directed mutagenesis (QuickChange; Stratagene) using a duplex of oligonucleotides (forward primer, CTGGGAGCTCACATGCCCCGCCCCGG CCCTCACCCTCATC) according to the manufacturer's instructions. The resulting plasmid was named pTK1C5-1C. The presence of the deletion and the absence of other mutations in *tk* were confirmed by sequencing. Two isolates of the recombinant viruses (TK1C5-1C.1 and TK1C5-1C.2) were generated from separate transfections by cotransfecting pTK1C5-1C with *tk*LTRZ1 DNA (13). *tk*LTRZ1 is a recombinant virus generated from strain KOS that has *lacZ* driven by the Moloney murine leukemia virus long terminal repeat (LTR) inserted into *tk* (8). Recombination between pTK1C5-1C and *tk*LTRZ1 yields viruses that have "white" plaques when stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which are easily distinguished from "blue" *tk*LTRZ1 plaques. White viruses were purified by limiting dilution (13, 16). *tk* genes from the purified isolates were sequenced to confirm the presence of the single-C deletion in the absence of other mutations.

A TK enzyme assay and PA both failed to detect active TK in TK1C5-1C-infected cells. Plaque autoradiography (PA) and measurements of enzymatic activity in cell lysates are sensitive techniques used to detect HSV TK activity. In our hands, the enzyme assay can detect approximately 0.4% of wild-type TK activity (17), and PA can detect approximately 0.25% of wild-type TK activity (15). We first asked whether we could detect TK activity in lysates of TK1C5-1C-infected cells using an

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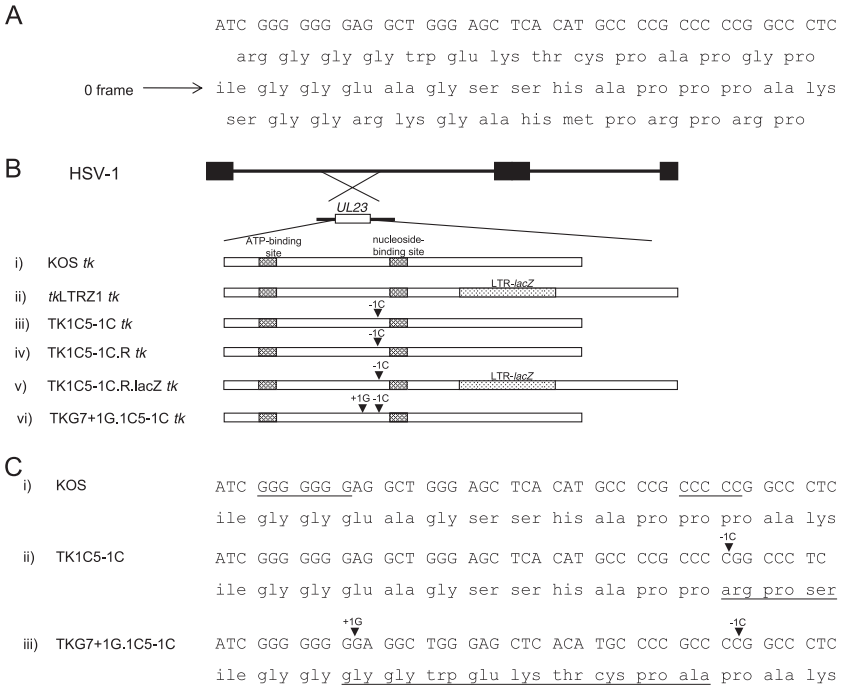

FIG. 1. (A) The top line represents the wild-type KOS *tk* sequence in the region of the G string. Below is a three-frame translation of this sequence, with the wild-type frame marked as the 0 frame. (B) The top two lines represent the HSV genome and the location of the *tk* gene (*UL23*). Below are schematic diagrams of the *tk* genes of the viruses used in this study: (i) KOS (cross-hatched boxes, functional domains of TK); (ii) *tk*LTRZ1 (*tk* with LTR-*lacZ* inserted into the PstI site [dotted box]); (iii) TK1C5-1C (*tk* with a single C deleted from a run of 5 Cs of KOS *tk* [arrow, site of mutation]); (iv) TK1C5-1C.R (TK1C5-1C that reactivated from latently infected mouse trigeminal ganglia); (v) TK1C5-1C.R.lacZ (*tk* with LTR-*lacZ* inserted into the PstI site, replacing the *tk* gene of TK1C5-1C.R); (vi) TKG7+1G.1C5-1C (*tk* with both a single C deleted from a run of five Cs and a single G inserted into to the G string of KOS *tk*). (C) (i) The top line represents the nucleotide sequence of KOS *tk* with the G string and the five Cs underlined. Below are the amino acids translated in the 0 frame. (ii) The top line represents the nucleotide sequence of TK1C5-1C *tk* (arrow, site of mutation). Below are the amino acids translated in the 0 frame. (iii) The top line represents the nucleotide sequence of TKG7+1G.1C5-1C *tk* (arrows, sites of mutations). Below are the amino acids translated in the 0 frame.

enzyme assay described previously (17), except that Vero cells were used and dried filters were digested in 4 ml of 75 mM sodium acetate, pH 5.0, and 10 mg cellulase for 60 min at 37°C in the scintillation tube. Scintillation fluid was added to the samples, which were then analyzed in a scintillation counter. Levels of active TK above background (*tk*LTRZ1-infected cell lysates) were not detected in cells infected with either TK1C5-1C.1 or TK1C5-1C.2 (data not shown). Next, with PA (2, 12, 13, 15), active TK was not detected in TK1C-1C-infected plaques (Fig. 2). In contrast, TK activity was readily detected

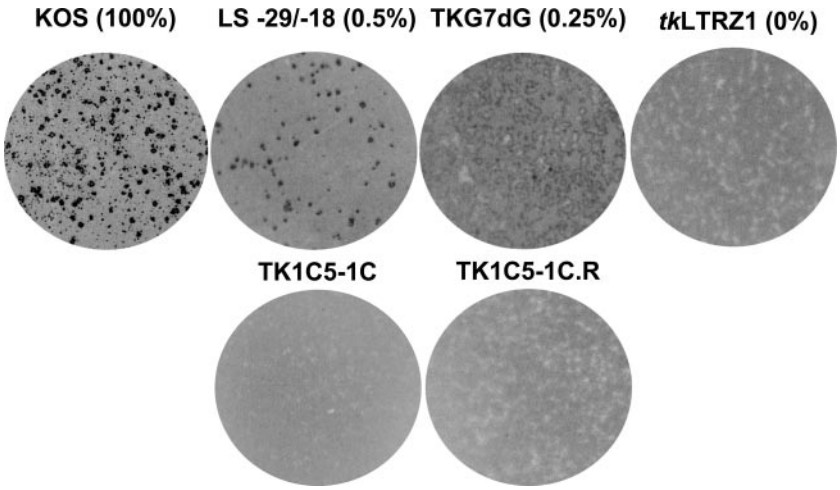

FIG. 2. PA of viruses. The top line shows the virus used, with the TK activity associated with that virus in parentheses. The images of the plates are shown below. The next line shows the viruses generated in this study. The images of the plates are shown below.

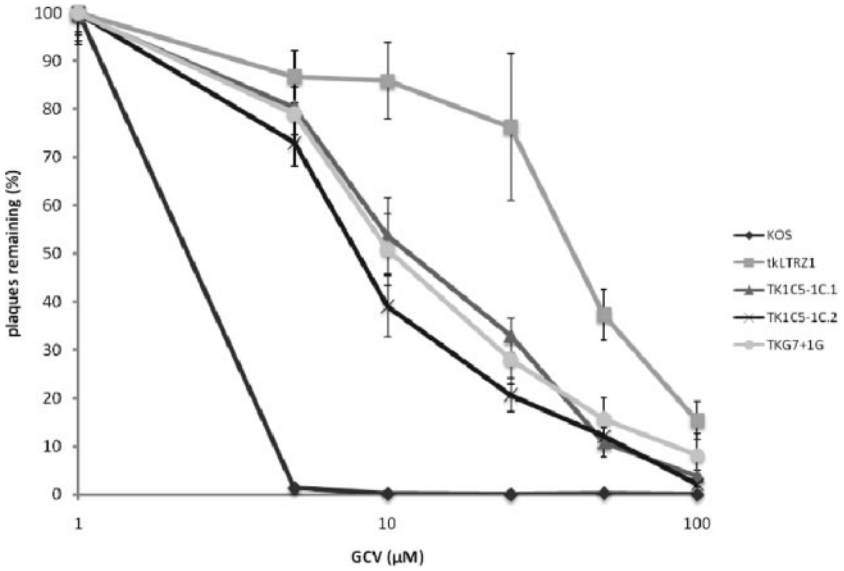

FIG. 3. Susceptibilities of viruses to GCV. Results are the averages of at least two separate experiments performed in triplicate. Error bars represent standard errors (SE). Where error bars are absent, the SE was too small to be visible on the graph.

with viruses LS-29/-18 and TKG7dG (Fig. 2), which have, respectively, been shown to synthesize 0.5% and 0.25% of wild-type TK (7, 15). Therefore, virus TK1C-1C generated less than 0.25% of wild-type TK activity.

Pharmacological assay to detect active TK. We considered the possibility that virus TK1C-1C may generate levels of active TK that were below the limits of detection of the above assays. It has been shown that susceptibility to antiviral drugs that require TK for activation is a sensitive method of detecting TK activity (4, 5, 7). We performed plaque reduction assays (PRA) to test the susceptibility of virus TK1C5-1C to the antiviral drug ganciclovir (GCV). For comparison, we included strains KOS (100% TK activity), TKG7+1G (0.5% [12]), and *tk*LTRZ1 (0% [8]). Assays were performed as previously described (4). As expected, KOS was most sensitive to GCV, and *tk*LTRZ1 was the least sensitive (Fig. 3). TK1C5-1C.1, TK1C5-1C.2, and TKG7+1G had similar GCV 50% effective doses (ED_{50}) (11 μM, 8 μM, and 10 μM, respectively), which were all lower than the GCV ED_{50} for *tk*LTRZ1 (40 μM). At 10 μM, 25 μM, and 50 μM, the differences in sensitivity to GCV of *tk*LTRZ1 compared to TK1C5-1C.1, TK1C5-1C.2, or TKG7+1G were highly significant (P [for each], <0.004 [Student's t test]). These data strongly suggest that like TKG7+1G, TK1C5-1C generated a low level of active TK. Although this assay was sensitive, we were unable to differentiate between the levels of TK expression from the TK1C5-1C viruses and TKG7+1G.

Translation of mutant amino acids between the G string and the TK1C5-1C mutation reduces TK activity. We hypothesized that a −1 ribosomal frameshift on the G string upstream of the TK1C5-1C mutation shifts the frame translated by the ribosome such that nine out-of-frame amino acids are translated before the wild-type frame is restored by the TK1C5-1C mutation (Fig. 1). Therefore, the TK activity of TK1C5-1C would be a product of the frameshifting efficiency on the G string (0.25%) and the effect of the non-wild-type amino acids. Importantly, the crystal structure of HSV TK shows that these residues are physically distant from the nucleoside and triphosphate binding sites (1, 24, 25). To estimate the effect of these amino acids on TK, we generated a virus with wild-type TK sequence, except for the amino acids between the G string and the TK1C5-1C mutation, by adding a G to the G string of a TK gene that already included the TK1C5-1C mutation (Fig. 1). Plasmid pTKG7+1G.1C5-1C was generated by site-directed mutagenesis (as described above) of pTK1C5-1C, with a duplex of oligonucleotides (forward primer, CTGGCTCCTCAT ATCGGGGGGGGAGGCTGGGAGCTC). The recombinant virus, TKG7+1G.1C5-1C, was generated from pTKG7+1G.1C5-1C, as described above. Viral TK activity in cell lysates was measured with the enzyme assay described above. Values from triplicate infections were averaged, and the enzyme activities in TKG7+1G.1C5-1C-infected cells were compared to serial dilutions of KOS-infected cell lysates. Using this assay we determined that virus TKG7+1G.1C5-1C had 35% (standard deviation, 3%) of the TK activity of KOS. Therefore, accounting for a −1 frameshifting efficiency of 0.25% and the effect of the out-of-frame amino acids on the enzyme activity, the TK activity of TK1C5-1C was estimated to be 0.09% of the wild-type level (35% of 0.25%).

Virus TK1C5-1C reactivated from latently infected mouse trigeminal ganglia, albeit at a low frequency. We had previously determined that levels of TK as low as 0.25% support reactivation of HSV from latently infected mouse trigeminal ganglia (virus reactivated from 2 of 28 ganglia) (15). To determine whether virus TK1C5-1C could reactivate from latency, male 8-week-old CD-1 mice (Charles River Laboratories) were infected via the cornea with 1×10^6 PFU of KOS or 7×10^7 PFU of TK1C5-1C.1, TK1C5-1C.2, or *tk*LTRZ1, as described previously (5, 21). At 30 days postinfection, the mice were sacrificed and the ganglia were harvested, enzymatically dissociated, and plated onto Vero cells, as described previously (21). Virus reactivated from 1 of 13 ganglia latently infected with TK1C5-1C.1, and no reactivation was observed from 13 ganglia

latently infected with TK1C5-1C.2. Virus reactivated from all of the ganglia latently infected with KOS (38 ganglia), and virus did not reactivate from any of the ganglia latently infected with *tk*LTRZ1 (46 ganglia). The low frequency of reactivation of TK1C5-1C was similar to that previously observed with viruses that express levels of TK of ≤0.5% of wild type (15).

Reactivation of TKC5-1C.R appeared not to be due to reversion and was most likely dependent on TK activity. The virus that reactivated from TK1C5-1C-infected ganglia (TK1C5-1C.R) was analyzed by PA (Fig. 2) and PRA (data not shown). In both assays, TK1C5-1C.R behaved like TK1C5-1C, indicating that the virus had not reverted to the wild-type TK phenotype. Sequencing confirmed that the TK open reading frame of TK1C-1C.R was identical to that of TK1C-1C. Additionally, given these observations and that TK1C5-1C virus was not handled during the incubation of the ganglia, contamination of the sample with virus TK1C5-1C was highly unlikely to be the source of TK1C5-1C.R.

Previous reports have shown that certain clinical isolates may be able to reactivate from latently infected mouse trigeminal ganglia in the absence of TK, suggesting the existence of alleles that may compensate for the lack of active TK (12, 17). To address whether TK1C5-1C.R reactivated from latency in a TK-dependent manner, we removed the capacity of the reactivated virus to synthesize active TK. To this end, we recombined the LTR-*lacZ* (from plasmid p*tk*LTRZ1 [8]) into TK1C5-1C.R to yield TK1C5-1C.R.lacZ (Fig. 1). This virus behaved similarly to *tk*LTRZ1 in a PRA (data not shown). Virus did not reactivate from any of the ganglia latently infected with TK1C5-1C.R.lacZ (14 ganglia). In contrast, the previous reports of TK-independent reactivation show it to be relatively efficient, with at least 30% of latently infected ganglia supporting reactivation (12, 17). Therefore, it seems unlikely that virus TK1C5-1C.R had acquired any mutations that conferred the ability to reactivate in the absence of TK. These observations are consistent with the notion that the very low levels of TK synthesized by TK1C5-1C were sufficient to support reactivation from latently infected mouse trigeminal ganglia.

Herein, we describe the characterization of a virus with a mutation that has been reported in drug-resistant HSV disease: a single-C deletion in a run of five Cs. We have estimated that this virus generates only 0.09% of wild-type TK activity and provided evidence that this is sufficient to support reactivation from latently infected mouse trigeminal ganglia, albeit weakly. This supports previous work suggesting that HSV-1 expresses much more TK than is required for ganglionic function, at least in the mouse (2, 12, 15).

It is important to note that TK1C5-1C generated levels of TK that could be detected only pharmacologically. Viruses with this mutation have previously been characterized as TK^- (10). However, the data presented in this paper suggest that it is possible that other viruses reported to be phenotypically TK^- based on PA (10; also reviewed in reference 11) may actually express low levels of TK.

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