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Characterization of a thymidine kinase-deficient mutant of equine herpesvirus 4 and in vitro susceptibility of the virus to antiviral agents

Walid Azab[a], Koji Tsujimura[b], Kentaro Kato[a], Jun Arii[a], Tomomi Morimoto[c], Yasushi Kawaguchi[c], Yukinobu Tohya[a], Tomio Matsumura[b], Hiroomi Akashi[a,*]

[a] Department of Veterinary Microbiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Epizootic Research Center, Equine Research Institute, Japan Racing Association, 1400-4 Shiba, Shimotsuke-shi, Tochigi 329-0412, Japan
[c] Department of Infectious Disease Control, International Research Center for Infectious Diseases, the Institute of Medical Science, the University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

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ABSTRACT

Equine herpesvirus 4 (EHV-4) is an important equine pathogen that causes respiratory tract disease among horses worldwide. A thymidine kinase (TK)-deletion mutant has been generated by using bacterial artificial chromosome (BAC) technology to investigate the role of TK in pathogenesis. Deletion of TK had virtually no effect on the growth characteristics of WA79ΔTK in cell culture when compared to the parent virus. Also, virus titers and plaque formation were unaffected in the absence of the TK gene. The sensitivity of EHV-4 to inhibition by acyclovir (ACV) and ganciclovir (GCV) was studied by means of a plaque reduction assay. GCV proved to be more potent and showed a superior anti-EHV-4 activity. On the other hand, ACV showed very poor ability to inhibit EHV-4 replication. As predicted, WA79ΔTK was insensitive to GCV. Although EHV-4 is normally insensitive to ACV, it showed >20-fold increase in sensitivity when the equine herpesvirus-1 (EHV-1) TK was supplied in trans. Furthermore, both ACV and GCV resulted in a significant reduction of plaque size induced by EHV-4 and 1. Taken together, these data provided direct evidence that GCV is a potent selective inhibitor of EHV-4 and that the virus-encoded TK is an important determinant of the virus susceptibility to nucleoside analogues.

1. Introduction

Equine herpesvirus 4 (EHV-4) is reported worldwide as an etiologic agent of respiratory disease, occasionally abortion and neonatal infection in horses. EHV-4 and its close relative equine herpesvirus 1 (EHV-1) are major pathogens of horses with considerable economic and veterinary importance (Allen and Bryans, 1986; Patel and Heldens, 2005; Slater et al., 2006). Both viruses have been classified into the *Alphaherpesvirinae* subfamily, genus *Varicellovirus* (Davison et al., 2009; Roizman, 1996). Although EHV-1 and EHV-4 are closely related, antigenic variations were recognized as early as 1959. More recently, molecular analysis and specific gene sequencing have revealed genetic differences between these two viruses. To date, control measures have proved inadequate, and although vaccines are available, their efficacy is controversial (Harless and Pusterla, 2006; Reed and Toribio, 2004).

EHV-4 genome manipulation with subsequent understanding of the viral gene functions has always been difficult due to the limited number of susceptible cell lines and the absence of small-animal models of the infection. Efficient generation of mutants of EHV-4 would significantly contribute to the rapid and accurate characterization of the viral genes. This problem has been solved recently by the cloning of the genome of EHV-4 as a stable and infectious bacterial artificial chromosome (BAC) without any deletions of the viral genes (Azab et al., 2009). Very low copy BAC vectors are the mainstay of present genomic research because of the high stability of inserted clones and the possibility of mutating any gene target in a relatively short time.

The study of thymidine kinase (TK)-deficient mutants of other herpesviruses has received considerable attention for many years. Deletion of this viral encoded enzyme in alphaherpesviruses, such as herpes simplex virus (HSV) types 1 and 2, bovine herpesvirus 1, and pseudorabies virus, resulted in a reduction in virulence and the rate of re-activation of latent viruses (Coen et al., 1989; Field and Wildy, 1978; Kit et al., 1985a; Mittal and Field, 1989; Slater et al., 1993; Stanberry et al., 1985). Previous reports of EHV-1, the close relative of EHV-4, TK-deficient mutants suggested that they are markedly less pathogenic than the wild-type virus, despite being able to replicate in the host (Cornick et al., 1990; Slater et al., 1993).

* Corresponding author. Tel.: +81 3 5841 5396; fax: +81 3 5841 8184.
E-mail address: akashih@mail.ecc.u-tokyo.ac.jp (H. Akashi).

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However, the role of TK in EHV-4 pathogenesis has not been yet elucidated.

Herpesvirus-encoded TKs also have an important role in the mode of action of several acyclic nucleoside analogues [e.g. acyclovir (ACV) and ganciclovir (GCV)] which are potent anti-herpetic drugs. The ultimately active metabolites of most nucleoside analogues are their triphosphates. Studies on the mode of action of ACV have shown that it is phosphorylated in infected cells by the virus-induced TK to ACV monophosphate (ACV-MP). After monophosphorylation, host cellular kinases convert ACV-MP to the di- and triphosphate (ACV-TP). ACV-TP is the active form that inhibits herpesvirus DNA polymerase (De Clercq, 1995; Field and Whitley, 2005; Morfin and Thouvenot, 2003). Although antiviral chemotherapy is a standard practice in the management of herpesvirus infection in humans, the veterinary use of antiviral drugs is relatively uncommon. Perhaps the most frequently reported use of antiviral drugs in veterinary medicine is for the treatment of feline herpesvirus 1 infections. However, there are some reports declared the efficacy of antiviral drugs against EHV-1, but not EHV-4, in vitro (De Clercq et al., 2006; Garré et al., 2007b; Hussein et al., 2008). Furthermore, the efficacy of 5-ethyldeoxyuridine and penciclovir against cercopithecine herpesvirus 1 was recently investigated (Focher et al., 2007). One of the objectives of this study was to compare the anti-EHV-4 activity of ACV and GCV by plaque reduction assay (PRA). GCV displayed a superior anti-EHV-4 activity, while ACV showed very poor ability to inhibit EHV-4 replication. Furthermore, the TK-negative mutant was resistant to the effect of the potent anti-herpetic drug GCV. These observations directed our attention toward investigating the role of TK-mediated phosphorylation in determining the susceptibility of EHV-4 to nucleoside analogues. To address this point, transfected fetal horse kidney (FHK) cells expressing EHV-1 TK gene was produced. Although EHV-1 seems to be less sensitive to ACV as compared with HSV-1, several recent reports have described the use of ACV for the treatment of horses during herpesvirus outbreaks (Garré et al., 2007a; Henninger, 2003; van der Meulen et al., 2003). The sensitivity of EHV-4 to ACV and GCV, TK-dependent drugs, in the EHV-1 TK-transformed FHK cells was investigated. We found that supplying EHV-1 TK in trans resulted in a marked increase in the sensitivity of EHV-4 to ACV.

2. Materials and methods

2.1. Viruses, cells and antiviral drugs

EHV-4 strain TH20p was plaque purified from the Japanese EHV-4 prototype strain TH20 (Kawakami et al., 1962; Maeda et al., 2004). The recombinant WA79, EHV-4 infectious BAC clone, was generated by the insertion of a *loxP*-flanked BAC vector into the intergenic region between genes 58 and 59 (Azab et al., 2009). EHV-1 strain 89c25, a virulent strain of EHV-1, was isolated from a race horse during an epizootic of EHV-1 respiratory infection in Japan in 1989 (Matsumura et al., 1992). Primary fetal horse kidney (FHK, within the fifth passage), Rabbit kidney (RK13), and human embryonic kidney (293) cells were propagated in Dulbecco's modified Eagle's medium (DMEM: Nissui) supplemented with 10% fetal calf serum (FCS) (JRH biosciences).

The antiviral compounds used were acyclovir (Sigma) and ganciclovir (LKT Laboratories, St. Paul, MN). The drugs were dissolved at 2 mg/ml in sterile distilled water and stored at −20 °C prior to dilution in cell culture medium.

2.2. Plasmids

The entire EHV-4 and 1 TK genes were polymerase chain reaction (PCR)-amplified using the primers listed in Table 1. The PCR products were digested with *Eco*RI and *Eco*RV and inserted into the expression vector pFLAG-CMV-2 (sigma), resulting in recombinant plasmids pFEH4TK and pFEH1TK, respectively. Correct amplification and insertion was confirmed by sequencing. Transfection of the pFEH4TK or the pFEH1TK plasmids into FHK cells was performed using TransIT-LT1 according to the Manufacturer's instructions (Mirus, Madison, WI).

2.3. Mutagenesis and generation of recombinant viruses

The EHV-4 BAC clone pYO03 has been generated previously (Azab et al., 2009). Mini F plasmid sequences flanked by *loxP* sites were inserted into the intergenic region between genes 58 and 59. DNA of pYO03 was manipulated by red recombination (Lee et al., 2001) in *E. coli* EL250 strain (Fig. 1), a kind gift from Dr. Neal G. Copeland. PCR primers were selected such that the recombination arms of 50 nucleotides (*nt*) enabled the substitution of *nt* 1 to 1058 of the EHV-4 TK gene by the kanamycin-resistant (Kan^R) gene amplified out of plasmid pEPkan-S, a kind gift from Dr. N. Osterrieder (Tischer et al., 2006). Primers used for the amplification of the Kan^R gene are listed in Table 1. PCR product was treated with *Dpn*I, to digest the template, and electroporated into EL250 containing pYO03. Kanamycin-resistant colonies were purified and screened by PCR, restriction enzyme digestion and DNA sequencing analyses to detect *E. coli* harboring the mutant pYOΔTK. The TK deletion mutant virus WA79ΔTK was reconstituted by transfection of pYOΔTK into 293 cells as described earlier (Azab et al., 2009). Three days later, the supernatant and cells were collected and used to infect confluent FHK cells. Viral plaques were observed 2 or 3 days after infection.

Table 1
Oligonucleotide primers used in this study.

Gene	Direction	Sequence
EHV-4 TK	Forward	*ACT***gaattc**ATGGCTGCTTGCGTACCCAC[a]
	Reverse	*AAT***gatatc**TCAGACGCCCATCTCCGCGT[a]
EHV-1 TK	Forward	*ACT***gaattc**ATGGCGGCTCGCGTACCTTC[a]
	Reverse	*AAT***gatatc**TCAGACGCCCATCTCGGCGT [a]
TK-Kan	Forward	ATGGCTGCTTGCGTACCCACGGGAGAAGCTCCACGAAGCGCCAGCGGAACAGGATGACGACGATAAGTAGGG[b]
	Reverse	TCAGACGCCCATCTCCGCGTTAAAGGTGCGTGCCGCCCGCTCTAAAGCAGCAACCAATTAACCAATTCTGATTAG[b]
TK	Forward	TTAGTGGTATTTACGACACC
	Reverse	TGCTTATAAGCCCCACAGGA
Kan	Forward	AGGATGACGACGATAAGTAGGG
	Reverse	CAACCAATTAACCAATTCTGATTAG

[a] Restriction enzyme sites are given in lower case bold letters; sequences in italics indicate additional bases which are not present in the EHV-4 sequence.
[b] Underlined sequences indicate the template binding region of the primers for PCR amplification with pEBkan-S.