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Inhibition of herpes simplex virus type 2 (HSV-2) viral replication by the dominant negative mutant polypeptide of HSV-1 origin binding protein

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Abstract

UL9-C535C, the *trans*-dominant negative mutant polypeptide of herpes simplex virus type 1 (HSV-1) UL9 origin binding protein, is a potent inhibitor of HSV-1 viral DNA replication. This study focused on testing whether HSV-1 UL9-C535C and a genetically engineered UL9-C535C-encoding HSV-1 recombinant virus CJ83193 could inhibit herpes simplex virus type 2 (HSV-2) infection. First, a stable cell line, R-C535C, expressing a high level of UL9-C535C in the presence of tetracycline and little or no UL9-C535C in the absence of tetracycline was established. The single step growth experiment showed that like HSV-1, the de novo synthesis of HSV-2 could be suppressed $\sim$1000-fold by UL9-C535C expressed in R-C535C cells in the presence of tetracycline. Secondly, compared with cells singly infected with HSV-2, co-infection of Vero cells with HSV-2 and CJ83193 reduced the replication efficiency of HSV-2 in co-infected cells by 30–40 fold in a single-step growth assay, which coincided with marked reduction in viral late gene expression, but not the expression of viral immediate-early genes. Taken together, in view of our recent demonstration that CJ83193 can serve as an effective vaccine in preventing HSV-1 infection in mice, one can generate a CJ83193-like HSV-2 recombinant virus that could potentially function as a new therapeutic class of recombinant viral vaccine against HSV-2 infection.

Keywords: HSV; Viral DNA replication; Dominant-negative polypeptide; Gene regulation; Tetracycline

1. Introduction

The major clinical significance of herpes simplex virus type 1 (HSV-1) and HSV-2 lies in their ability to cause acute primary infection and to reactivate periodically from latency causing recurrent infection. Although HSV infections are often asymptomatic, they can manifest clinically; for example, oral-facial infections, genital herpes, neonatal herpes, keratoconjunctivitis, and herpes encephalitis (reviewed by Stanberry et al., 2000; Whitley et al., 1998).

HSV replicates in epithelial cells and establishes life-long latent infection in neuronal cell bodies

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within the sensory ganglia of infected individuals. During productive infection, HSV gene expression falls into three major classes based on the temporal order of their expression. The three classes have been designated immediate-early, early, and late with late genes being further divided into two groups, γ1 and γ2 (reviewed by Roizman and Sears, 1996). In contrast to the expression of immediate-early and early genes, which do not require viral DNA synthesis, expression of late genes depends on de novo viral DNA synthesis. Specifically, expression of γ1 genes is enhanced by viral DNA replication, whereas expression of γ2 genes is inhibited in the absence of viral DNA synthesis.

Although there exists some significant differences between HSV-1 and HSV-2, such as their host range and their ability to turn off host protein synthesis in virus-infected cells, these two viruses share significant DNA sequence homology (Dolan et al., 1998; McGeoch et al., 1986, 1988). The genome of HSV-1 and, probably that of HSV-2 encode more than 84 distinct proteins (reviewed by Whitley et al., 1998; Ward et al., 2000) and contain three origins of DNA replication, one designated oriL, and the other two designated oriS (Fig. 1A). HSV-1 oriS contains three highly homologous elements, named sites I, II, and III, which interact specifically with the origin-binding protein encoded by the HSV-1 UL9 gene (Dabrowski and Schaffer, 1991; Deb and Deb, 1989; Elias and Lehman, 1988; Elias et al., 1986; Koff and Tegtmeyer, 1988; Olivo et al., 1988; Weir and Stow, 1990; Weir et al., 1989). Interestingly, the HSV-2 origins of replication contain core elements that are identical to the HSV-1 UL9 binding sites I and III (Fig. 1B; Lockshon and Galloway, 1986).

HSV-1 UL9 protein consists of 851 amino acids (McGeoch et al., 1988) with its DNA binding domain residing within the C-terminal amino acids 535–851 (Deb and Deb, 1991; Hazuda et al., 1992). The binding of wild-type UL9 protein to the origins of DNA replication is essential for viral DNA replication. It has been shown that the UL9 C-terminal DNA binding domain possesses a DNA binding affinity similar to that of wild-type UL9 protein for the HSV-1 origins of replication (Hazuda et al., 1991). When expressed at high levels, the UL9 DNA binding domain, for example, the C-terminal amino acids 535–851 of UL9 (UL9-C535C), can exert a potent dominant-negative effect on HSV-1 DNA replication (Malik and Weller, 1996; Perry et al., 1993; Stow, 1992; Stow et al., 1993; Yao and Eriksson, 1999b).

Recently, we constructed a novel class of HSV-1 recombinant virus (CJ83193) encoding UL9-C535C under the control of the tetracycline operator (tetO)-containing hCMV major immediate-early promoter (Fig. 1A Yao and Eriksson, 1999a). As the gene expression from the tetO-containing hCMV major immediate-early promoter can be tightly suppressed by tetracycline repressor, tetR (Yao and Eriksson, 1999b; Yao et al., 1998), CJ83193 expresses little to no UL9-C535C in tetR-expressing cells, and high levels of UL9-C535C in non-tetR expressing cells or tetR-expressing cells in the presence of tetracycline. It was shown that compared with its parental non UL9-C535C-expressing virus, 7134 (Fig. 1A), the de novo production of CJ83193 is reduced by more than six orders of magnitude in non-tetR expressing cells. Moreover, UL9-C535C peptides expressed from the CJ83193 genome can inhibit the replication of wild-type HSV-1 in co-infected cells by 100–200-fold.

In light of the dominant-negative effect of UL9-C535C on HSV-1 replication, and the presence of HSV-1 UL9 binding sites within the HSV-2 origins of replication, the focus of this study was to test whether UL9-C535C can function as a therapeutic polypeptide to inhibit HSV-2 infection.

2. Inhibition of HSV-2 viral replication by UL9-C535C

To compare the efficiency of the dominant-negative effect of UL9-C535C on de novo viral production of HSV-2 relative to HSV-1, we established a double stable line R-C535C by stable transfection of a tetR-expressing osteosarcoma cells U2CEP4R-11 with plasmids pcDNA3 and pcmvtetOUL9-C535C that encodes UL9-C535C under the control of the tetO-containing hCMV

major immediate-early promoter (Yao and Eriksson, 1999b). The Western blot analysis in Fig. 2 showed that while significant amounts of UL9-C535C were expressed in R-C535C cells grown in the presence of tetracycline, no UL9-C535C was detected in cell extract prepared from R-C535C cells grown in the absence of tetracycline. Thus, the expression of UL9-C535C can be effectively regulated by tetracycline in R-C535C cells.

To investigate whether high-levels of UL9-C535C expression in R-C535C cells could inhibit HSV-2 infection, single step growth experiments in the presence and the absence of tetracycline were performed (Table 1). As shown, compared with infected cells that had not been treated with tetracycline, the presence of tetracycline led to about 1000- and 700-fold reduction in HSV-2 and HSV-1 viral synthesis in R-C535C cells, respectively. Given that tetracycline treatment had no

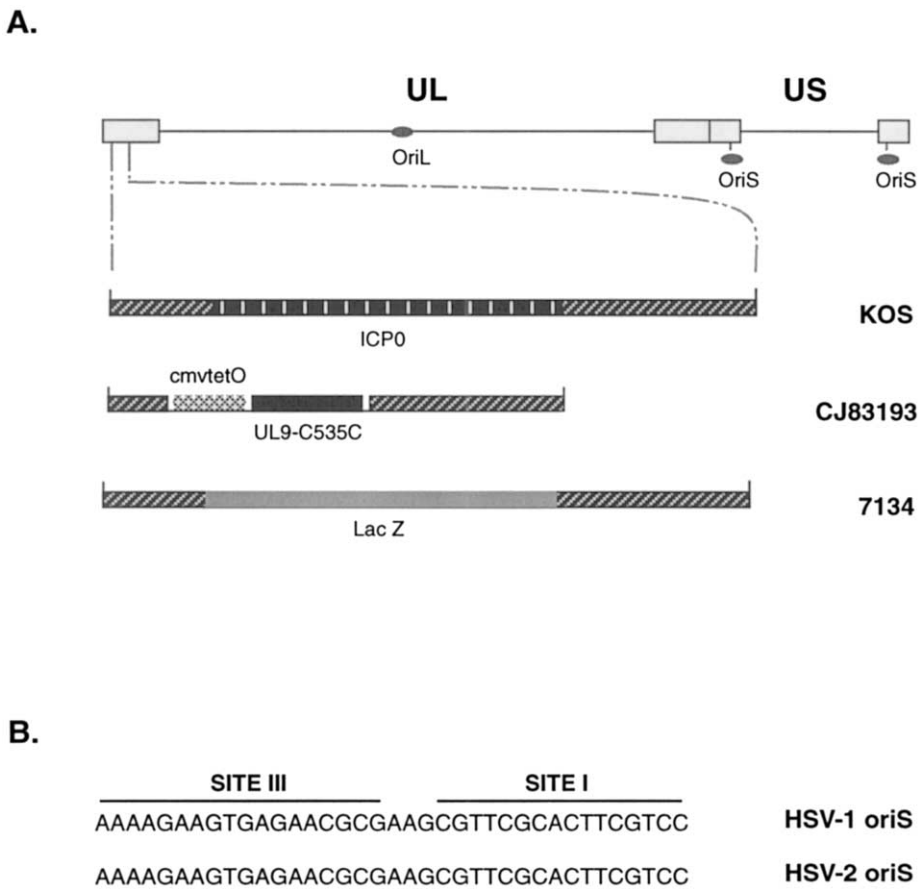

Fig. 1. (A) A schematic diagram of genomes of the wild-type HSV-1 and HSV-2, the dominant-negative HSV-1 recombinant CJ83193, and a HSV-1 ICP0 null mutant, 7134, the parental virus of CJ83193. The top diagram shows the HSV genome, indicating the unique long (UL) region, the unique short (US) region, the inverted repeat regions (open boxes), and the three origins of DNA replication (gray oval). The diagram beneath that of the HSV genome represents an expanded Sac I–Pst I DNA fragment containing the ICP0 open reading frame with flanking sequences in wild-type HSV-1, strain KOS, the DNA sequences encoding the UL9-C535C under the control of the tetO-bearing hCMV major immediate-early promoter with the ICP0 flanking sequences in CJ83193, and the lac Z gene with ICP0 flanking sequences in 7134 (Cai and Schaffer, 1989). (B) The top line shows site I and site III DNA sequences within the HSV-1 OriS that interact specifically with HSV-1 DNA replication origin-binding protein UL9. The bottom line shows DNA sequences within the HSV-2 oriS that are identical to the HSV-1 UL9 binding sites I and III.

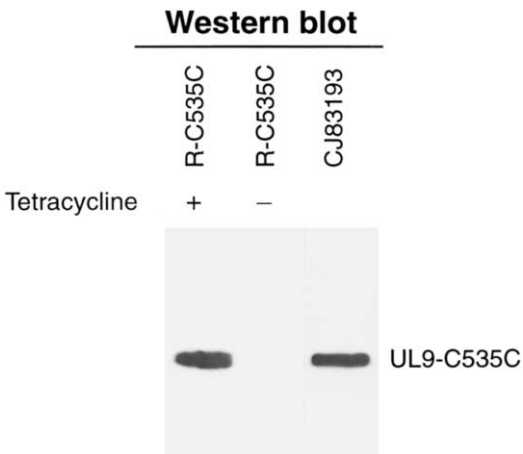

Fig. 2. Regulation of UL9-C535C expression by tetracycline in R-C535C cells. R-C535C cells were seeded at 2.5×10^6 cells per 100 mm dish. At 24 h post-seeding, cells in duplicate were grown in either the absence or the presence of tetracycline at 2.5 μg/ml, and cell extracts were prepared 20 h later. Proteins in cell extracts were resolved on SDS-PAGE followed by Western blot analysis with an anti-UL9 specific polyclonal antibody according to the protocol described previously (Yao and Schaffer, 1995). As a positive control, proteins from CJ83193-infected Vero cell extracts (83193) were also loaded on the same gel.

effect on HSV-1 and HSV-2 infection in tetR-expressing and UL9-C535C negative line R-CDNA3 (data not shown), these results demonstrate that the HSV-1 UL9-C535C can effectively inhibit HSV-2 viral infection.

3. *Trans*-dominant negative effect of CJ83193 on wild-type HSV-2 infection

Previously, it was shown that CJ83193 can inhibit wild-type HSV-1 viral production in co-infected cells by over 100-fold. To test if similar effects can also be observed on HSV-2 viral production, we performed a co-infection assay (Table 2). The results in Table 2 showed that compared with cells singly infected by HSV-2, co-infection of Vero cells with HSV-2 and CJ83193 resulted in an average of 34-fold lower HSV-2 viral production. This observed reduction in HSV-2 synthesis was not detected in Vero cells co-infected with HSV-2 and 7134, or HSV-2 and d2, an HSV-1 replication-defective ICP4 deletion mutant (DeLuca and Schaffer, 1988), suggesting that inhibition in HSV-2 viral synthesis in HSV-2 and CJ83193 co-infected cells is UL9-C535C specific.

To test this further, Vero cells in duplicate were independently infected in the same manner as the

Table 1
Inhibition of HSV-1 and HSV-2 viral replication by HSV-1 UL-9-C535C expressed in R-C535C cells in the presence of tetracycline

Viruses	Viral titer ($\times 10^4$ PFU/ml)	
	Tetracycline (+)	Tetracycline (−)
HSV-1	5.03 ± 0.38	3416.7 ± 315.6
HSV-2	0.08 ± 0.02	91.7 ± 33.3

R-C535C cells were seeded at about 2.5×10^5 cells per 60 mm dish. A 20 h post-seeding, extracellular medium was removed and fresh growth medium was then added to the dishes either with no tetracycline (−) or with tetracycline (+) at 2.5 μg/ml. Fifty-five hours after addition of tetracycline, dishes of cells in triplicate were independently infected with either wild-type HSV-1 strain KOS, or wild-type HSV-2 strain 186 syn^+ at an MOI of 3 PFU per cell. Infections were carried out in either the absence (−) or presence of tetracycline (+) at 2.5 μg/ml. Cells were harvested 18 h post-infection and viral titers were determined by standard plaque assay on Vero cell monolayers and presented as the mean value ± S.D. of viral titers of three independent infections.

Table 2
Inhibition of HSV-2 infection by HSV-1 dominant-negative recombinant virus CJ83193 on Vero cell monolayers

Viral titer ($\times 10^5$ PFU/ml)			
HSV-2	HSV-2 + 7134	HSV-2 + CJ83193	HSV-2 + d2
74.7 ± 7.4	95.67 ± 4.5	2.17 ± 0.1	40.0 ± 7.0

Vero cells were seeded at 3×10^5 cells per 60 mm dish. At 48 h post-seeding, cells in triplicate were independently infected with (1) wild-type HSV-2 at MOI of 1 PFU per cell; (2) HSV-2 at MOI of I PFU per cell and 7134 at MOI of 3 PFU per cell; (3) HSV-2 at MOI of 1 PFU per cell and CJ83193 at MOI of 3 PFU per cell; and (4) HSV-2 at MOI of 1 PFU per cell and an HSV-1 ICP4 deletion mutant, d2, at MOI of 3 PFU per cell. Cells were harvested at 18 h post infection. Virus yields were determined by standard plaque assay on Vero cell monolayers and presented as the mean value ± S.D. of viral titers of these independent infections.

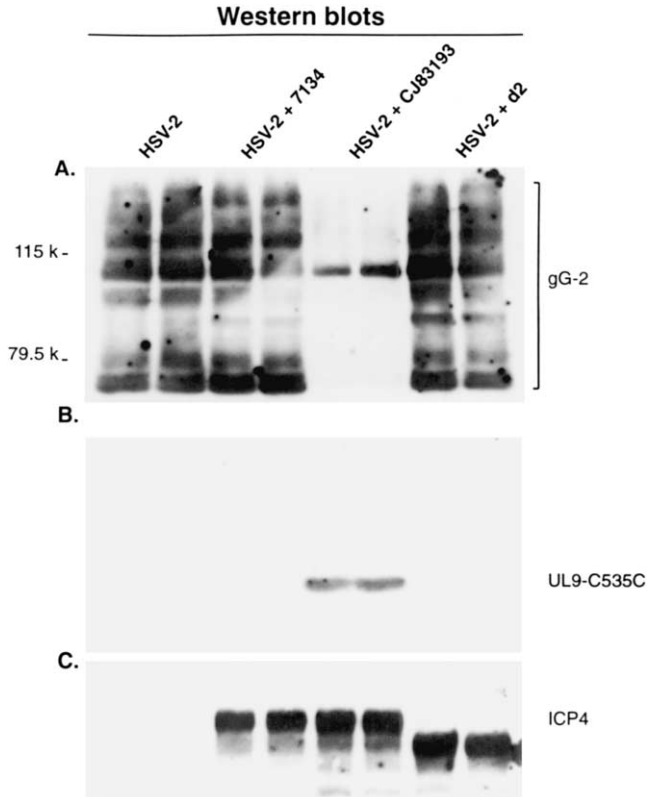

Fig. 3. Inhibition of HSV-2 late gene expression by UL9-C535C in HSV-2 and CJ83193 co-infected cells. Vero cells were seeded at 7×10^5 cells per 60 mm dish. At 48 h post-seeding, cells in duplicate dishes were infected with (1) wild-type HSV-2 at MOI of 1 PFU per cell; (2) HSV-2 at MOI of 1 PFU per cell and 7134 at MOI of 3 PFU per cell; (3) HSV-2 at MOI of 1 PFU per cell and CJ83193 at MOI of 3 PFU per cell; and (4) HSV-2 at MOI of 1 PFU per cell and d2 at MOI of 3 PFU per cell. Infected cell extracts were prepared at 16 h post-infection as described previously (Yao and Eriksson, 1999a). Proteins in infected cell extracts were analyzed by SDS-PAGE and immunoblotted with a monoclonal antibody specific for gG2 (Fitzgerald Industries Internation, Concord, MA), a polyclonal antibody specific for UL9, and a monoclonal antibody specific for ICP4 (Fitzgerald Industries Internation, Concord, MA), respectively.

experiments described in Table 2. Proteins from infected cell extracts in similar amounts were resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with an anti-HSV-2 gG-2 specific monoclonal antibody, and an anti-HSV-1 UL9 specific polyclonal antibody (Olivo et al., 1989). The gG-2 glycoprotein is first synthesized as a cotranslationally glycosylated intermediate with a molecular weight of about 104 kDa, which then undergoes a post translational cleavage, yielding two polypeptides with molecular weights of about 31 and 72 kDa, respectively. While the 31-kDa polypeptide is rapidly secreted from infected cells, the 72-kDa polypeptide is further glycosylated (Balachandran and Hutt-Fletcher, 1985; Su et al., 1987). The gG-2 gene belongs to HSV-2 delay-early gene family whose expression is enhanced by viral DNA replication. Thus, if the expression of UL9-C535C leads to inhibition in HSV-2 viral DNA replication in HSV-2 and CJ83193 co-infected cells, the gG-2 expression would be reduced in co-infected cells relative to cells singly infected by HSV-2. As shown in Fig. 3A, although similar levels of gG-2 were detected among cells infected by HSV-2, HSV-2 and 7134, and HSV-2 and d2, gG-2 expression was significantly reduced in HSV-2 and CJ83193 co-infected

cells as evidenced by the absence of the 72 kDa gG-2 polypeptide (Fig. 3A). Fig. 3B provides a proof that UL9-C535C was expressed specifically in HSV-2 and CJ83193 co-infected cells. Given that about equal amounts of ICP4 were present in cells co-infected by HSV-2 and 7134, HSV-2 and CJ83193, and HSV-2 and d2 (Fig. 3C), it is likely that the marked reduction in gG-2 synthesis in HSV-2 and CJ83193 co-infected cells was due to the *trans*-inhibitory effect of UL9-C535C on HSV-2 viral DNA replication.

During the past three decades, two major classes of HSV recombinant viruses have been generated, replication-defective viruses; and neuroattenuated HSV mutants (reviewed by Roizman, 1996; Whitley, 1993). The efficacy of these viruses as a potential viral vaccine against HSV infection has been evaluated in various animal models and the results are promising (reviewed by Stanberry et al., 2000; Whitley et al., 1998). For example, it was shown that HSV-1 ICP27 or ICP8 replication-defective recombinants could induce both humoral and cellular immunity at a level comparable with that of wild-type HSV-1, and mice immunized with these replication-defective recombinants were protected against wild-type HSV-1 infection (Nguyen et al., 1992; Morrison and Knipe, 1994). Recently, we have shown that, like the replication-defective ICP27 recombinant virus, CJ83193 can serve as an effective vaccine against wild-type HSV-1 infection in a mouse ocular model (manuscript in preparation). Moreover, we demonstrate that CJ83193 can also directly block acute wild-type HSV-1 infection in mice co-inoculated with HSV-1 and CJ83193 (manuscript in preparation). Collectively, on the basis of demonstrated characteristics of CJ83193 and the potent dominant-negative effect of HSV-1 UL9-C535C on HSV-2 viral replication, one can generate a replication-defective and UL9-C535C-expressing HSV-2 recombinant that can potentially serve as a new class of recombinant viral vaccine against HSV-2 infection. As the expression of UL9-C535C can inhibit HSV viral replication, this new class of recombinant HSV viral vaccines exhibits a unique safety feature capable of reducing the potential outbreak of the vaccine virus in vaccinated populations and recombination of the vaccine virus with its wild-type virus in the host.

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