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Antagonizing activity of vaccinia virus E3L against human interferons in Huh7 cells

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ABSTRACT

The E3L protein of vaccinia virus (VV) is well known for its capacity to evade cellular innate antiviral immunity related to interferon (IFN), for example PKR and RNaseL mediated antiviral activities. However, due to the limited range of cells that support VV E3L deletion mutant replication, the full capacity of E3L inhibiting the innate immune response induced by IFNs remains to be examined. In this report, the inhibition activity of VV E3L against a wide spectrum of human IFNs, including type I IFNs (12 IFN-α subtypes, IFN-β, and IFN-ω), and type II IFN (γ), was comparatively examined using the Copenhagen strain E3L deletion mutant and its revertant control virus in a human hepatoma cell line, Huh7. Deletion of the E3L open reading frame rendered the mutant VV sensitive to all types of IFNs, while the revertant VV was strongly resistant to these treatments. Furthermore, we show that the inhibition of VV E3L deletion mutant by IFN occurs at the stage of intermediate gene translation, while the expression of early genes and transcription of intermediate genes are largely unaffected. Using specific siRNAs to suppress the classical IFN-induced antiviral pathways, we found that PKR is the key factor modulated by E3L, while the RNaseL and MxA pathways play limited roles in this Huh7 cell system. Thus, our data demonstrates that VV E3L can mediate strong inhibition activity against all human type I and type II IFNs, mainly through modulation of the PKR pathway in Huh7 cells.

Introduction

Interferons (IFNs) play a pivotal role in innate immunity against viral infections. In mammals, IFNs that mediate antiviral activity include: type I IFNs, composed of several IFN-α subtypes, IFN-β, and IFN-ω and a single member of type II IFNs, IFN-γ. The more recent described IFNs-λ1, λ2, and λ3 (alternatively known as IL-29, IL-28A/B), are induced by viral infections similar to type I IFNs, and have been shown to exhibit antiviral and antiproliferative activities *in vitro* and in response to poxvirus infection *in vivo* (Meager et al., 2005; Onoguchi et al., 2007; Bartlett et al., 2005). Both type I and type II IFNs induce the JAK/STAT signaling pathway in all nucleated cell types and transcriptionally regulate several interferon stimulated genes (ISGs) that result in the production of antiviral proteins, including PKR, 2′–5′ OAS, and myxovirus resistance protein (MxA). All IFN-α subtypes are similar in structure and share a common receptor (IFNAR1/2), and can elicit antiviral activity, but may also vary in their ability to activate signaling pathways in certain cell types, and are likely to induce different genes (Foster and Finter, 1998; Pestka et al., 2004; Pfeffer, 1997; Yano et al., 2006). Thus, it is entirely possible that different IFN subtypes may initiate different degrees of antiviral activity.

Millions of years of virus and host co-evolution have led viruses to develop a wide variety of strategies to antagonize IFN activity. Of interest, a dsRNA binding protein encoded by vaccinia virus (VV), E3L, is well known for its capacity to block IFN induction, signaling, and activity. IFN inducible dsRNA-dependent Protein Kinase R (PKR) and 2′–5′-oligoadenylate synthetase (2′–5′ OAS) are activated in the presence of dsRNA which leads to the inhibition of translation initiation through eIF2α phosphorylation and the degradation of mRNA by ribonuclease RNaseL, respectively (Garcia et al., 2006; Carroll et al., 1997). Competitive binding of E3L to dsRNA inhibits the activation and overall function of both PKR and 2′–5′ OAS and has been associated with the inhibition of IRF-3 phosphorylation and the suppression of IFN-β induction (Chang et al., 1992; Davies et al., 1993; Xiang et al., 2002; Smith et al., 2001). The similar strategy to antagonize IFN antiviral function through dsRNA binding is also used by several other vertebrate viruses such as reovirus σ3, HSV-1 US11, avian reovirus σA, and influenza virus NS1 (Imani and Jacobs, 1988; Jacobs and Langland, 1998; Langland et al., 2006; Lu et al., 1995).

Deletion of E3L (VVΔE3L) is associated with a highly restricted host range. For example, in comparison with the wild-type virus, VVΔE3L is unable to replicate in Vero, murine L929, or HeLa cells (Beattie et al., 1996; Langland and Jacobs, 2002; Shors et al., 1998, 1997). It is well known that PKR and RNaseL, both of which can be regulated by IFNs, play major roles in determining the highly restricted host range of a VV E3L deletion mutant (Langland and Jacobs, 2002; Beattie et al., 1996; Xiang et al., 2002). However, the interplay between E3L and IFN-induced cellular responses (which involves more than 300 genes) in

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relation to virus replication has not been well characterized due to the limited cell lines available for such analysis. Previous studies on IFN sensitivity of a VV E3L deletion mutant were based on HeLa (Langland and Jacobs, 2004) or L929 cells (Beattie et al., 1995a,b; Xiang et al., 2002). However, in these cells, the replication of a VV E3L deletion mutant is defective. A VV E3L deletion mutant can replicate in rabbit kidney, RK-13 cells (Chang et al., 1995), and was shown to be sensitive to treatment with rabbit IFN-α in this cell line (Shors et al., 1997). Nonetheless, it is unlikely that the true capacity of E3L inhibiting human IFN antiviral activity can be analyzed using these cell lines and the interplay between E3L and IFN-induced antiviral signaling remains unclear.

In this study, we found that the VV E3L deletion mutant can efficiently replicate in Huh7 cells. Using this cell line, we show that E3L can mediate potent inhibition on antiviral activity induced by type I and type II IFNs through modulation of the PKR pathway.

Results

The VV E3L deletion mutant can replicate efficiently in the human hepatoma cell line Huh7

The E3L deletion mutant (Cop-ΔE3L) and its revertant (Cop-ΔE3L-Rev) were constructed based on the Copenhagen strain as described in Materials and methods. The only difference between the E3L deletion mutant and revertant viruses is the absence and presence of the *E3L* ORF, respectively (Fig. 1a). We confirmed the absence and presence of the E3L protein in the E3L deletion mutant and revertant viruses by Western blot (data not shown). The difference between the revertant virus and the wild-type Copenhagen strain is the inclusion of gpt (for positive selection) and EGFP for easy plaque identification and orientation of the E3L ORF. The inclusion of gpt and EGFP has no effect on virus replication and IFN sensitivity (data not shown). As shown in Fig. 1a, the mRNA expression of two adjacent ORFs, *E2L* and *E4L*, was not affected by deletion of *E3L*. The replication capability of the E3L deletion mutant was tested in six human cell lines and compared to BHK21. The human hepatoma cell line Huh7, was the only cell line to efficiently support replication of the E3L deletion mutant, while the E3L deletion mutant completely failed to replicate in MRC5, AD293, and HeLa cells (Fig. 1b).

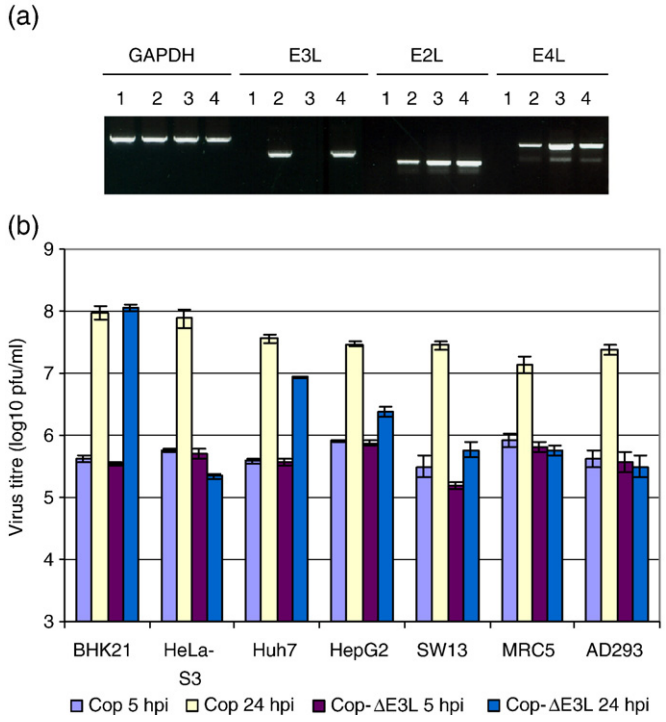

Fig. 1. Confirmation of the deletion of VV E3L in the E3L deletion mutant and the expression of E3L in the revertant control viruses. (a) Total RNA was extracted from BHK21 cells infected with Cop-ΔE3L, Cop-ΔE3L-Rev, and wild-type VV Cop. RT-PCR analysis confirm *E3L* mRNA expression is absent in the BHK21 cell control (lane 1), and in the E3L deletion mutant (Cop-ΔE3L) (lane 3), but is present in wild-type VV Cop (lane 2) and in the revertant control (Cop-ΔE3L-Rev) (lane 4). Deletion of *E3L* did not affect the mRNA expression of adjacent ORFs, *E2L* and *E4L*. (b) Human hepatocellular carcinoma, Huh7 cells, efficiently support Cop-ΔE3L replication. Confluent cell monolayers were infected at a MOI of 1 with wild-type VV Copenhagen strain (Cop) and Cop-ΔE3L and harvested 5 and 24 hpi. Virus titres ($\log_{10}$ pfu/ml) were measured in triplicate. Error bars indicate standard error of the mean.

VV E3L is a potent inhibitor to all human type I and type II interferons in Huh7 cells

Huh7 cells have been reported to be responsive to human type I IFNs (Blight et al., 2002). We confirmed the Huh7 cell line we used in this study is responsive to both type I and type II IFNs by activation of STAT1 by phosphorylation at two residues, tyrosine 701 and serine 727 (Supplementary Fig. 1).

To analyze the inhibition activity of the E3L protein on human IFNs in Huh7 cells, Cop-ΔE3L and its revertant were comparatively analyzed for their sensitivity to both type I and type II IFNs (including the 12 IFN-α subtypes, IFN-β, IFN-ω, and IFN-γ) (Fig. 2). As shown in Fig. 2a, Cop-ΔE3L-Rev is resistant to treatment of all type I and type II IFNs tested, depicted by the consistent pattern of replication of the revertant virus in the presence of all IFN species. Generally, a slight decrease in Cop-ΔE3L-Rev replication is seen with an IFN dose of 200 to 2000U/ml of type I and type II IFN species.

In contrast, deletion of the *E3L* gene results in a dramatic increased sensitivity to all human IFN species: IFN-β, IFN-ω, IFN-γ, and the IFN-α subtypes. As illustrated in Fig. 2b, replication of Cop-ΔE3L is inhibited in the presence of type I and type II IFNs in a dose-dependent manner. Even at a low dose of 20U/ml, IFN species demonstrated potent inhibition, decreasing virus replication by 100-fold, seen in IFN-β, IFN-ω, and IFN-γ treated Huh7 cells. Among all IFNs tested, the most potent IFN species was IFN-α14, which inhibited replication of Cop-ΔE3L by 1000-fold at its highest dose of 2000U/ml. Following IFN-α14, were type I IFNs: IFN-α2a, IFN-β, and type II IFN-γ, each of which inhibited replication of Cop-ΔE3L between 100 to 1000-fold. In contrast, the least effective at inhibiting viral replication was IFN-α7, followed by IFN-α5.

Cop-ΔE3L IFN sensitivity is mediated by PKR

To further characterize the mechanism of IFN resistance of Cop-ΔE3L-Rev and sensitivity of Cop-ΔE3L mutant viruses (Fig. 2), we examined the roles of each of the three main IFN-induced classical antiviral pathways: PKR, 2′–5′ OAS/RNaseL, and MxA pathways. Since IFNs transcriptionally induce PKR, to investigate the PKR pathway, we first monitored any change in endogenous PKR protein levels in type I (leukocyte, -β,-ω) and type II (γ) IFN-treated Huh7 cells in the absence and presence of virus infection with Cop-ΔE3L-Rev and Cop-ΔE3L. As shown in Fig. 3a, the level of PKR in Cop-ΔE3L-Rev infected Huh7 cells is comparable to the level of PKR in the cell control in the absence of IFN treatment. However, the level of total PKR appears to be reduced in Huh7 cells infected with Cop-ΔE3L in the absence of IFN treatment. Treatment with type I (leukocyte, -β,-ω) and type II (γ) IFNs slightly enhanced the level of endogenous PKR in the absence of virus infection. Even in the presence of both type I and type II IFNs, total PKR seems to be slightly reduced in all Cop-ΔE3L infected cells, whereas in all Cop-ΔE3L-Rev infected cells, there is no difference in PKR in the absence or presence of IFN treatment. It is important to note that the antibody used in this study recognizes endogeneous PKR protein and was