Herpes simplex virus-1 infection causes the secretion of a type I interferon-antagonizing protein and inhibits signaling at or before Jak-1 activation

Karen E. Johnson, David M. Knipe *

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA

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**A B S T R A C T**

Host cells respond to viral infection by the production of type I interferons (IFNs), which induce the expression of antiviral genes. Herpes simplex virus 1 (HSV-1) encodes many mechanisms that inhibit the type I IFN response, including the ICP27-dependent inhibition of type I IFN signaling. Here we show inhibition of Stat-1 nuclear accumulation in cells that express ICP27. ICP27 expression also induces the secretion of a small, heat-stable type I IFN antagonizing protein that inhibits Stat-1 nuclear accumulation. We show that the inhibition of IFN-induced Stat-1 phosphorylation occurs at or upstream of Jak-1 phosphorylation. Finally, we show that ISG15 expression is induced after IFN treatment in mock-infected cells, but not cells infected with WT HSV-1 or ICP27 + HSV-1. These data suggest that HSV-1 has evolved multiple mechanisms to inhibit IFN signaling not only in infected cells, but also in neighboring cells, thereby allowing for increased viral replication and spread.

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**I n t r o d u c t i o n**

One of the first lines of defense that is activated upon infection of a host with a pathogen is the interferon (IFN) response. Type I IFNs (α, β, ω, γ) are a family of antiviral cytokines induced in most cell types by viral infection or the presence of double-stranded RNA and acts in an autocrine and paracrine manner to establish an antiviral state in host cells (Sato et al., 2000). Type II IFN (γ) is a pro-inflammatory cytokine induced in activated T cells and natural killer cells (Schiller et al., 2006). Though there are distinct similarities in the signaling pathways activated by each type of IFN, there are also some key differences. Each family of IFN binds to a distinct heterodimeric receptor (Kotenko et al., 2003; Platanias and Colamonti, 1992; Platanias, Uddin, and Colamonti, 1994; Shepphard and York, 1990), which causes the activation of Janus kinases (Jaks) by phosphorylation. The kinases Jak-1 and Tyk-2 are activated in the case of type I IFN, and Jak-1 and Jak-2 for type II IFN (Darnell, Kerr, and Stark, 1994; David et al., 1993; Platanias, Uddin, and Colamonti, 1994). The Jaks phosphorylate signal transducers and activators of transcription (Stats)-1 and -2, in type I IFN signaling, and only Stat-1 after exposure to IFNγ (Platanias, Uddin, and Colamonti, 1994; Schindler et al., 1992; Uddin, Chamdin, and Platanias, 1995). Once activated by phosphorylation, Stat-1 either homodimerizes (IFNγ) or forms a complex with Stat-2 and with interferon regulatory factor 9 (IFNα/β) (Bandyopadhyay et al., 1995; Kessler et al., 1990; Ramana et al., 2002). These complexes translocate into the nucleus and bind specific DNA elements, interferon stimulated response elements (ISREs, type I signaling) or gamma activated sequences (GASs, type II signaling), to activate transcription of interferon stimulated genes (ISGs). ISGs contribute to the pro-inflammatory or antiviral state and include RNase L, which degrades viral and cellular RNAs (Dong and Silverman, 1995; Kerr and Brown, 1978) and PKR, which inhibits protein synthesis by phosphorylating the translation initiation factor eIF2a (Der et al., 1998; Samuel, 1979a,b).

Viruses have evolved mechanisms to evade or counteract the effects of IFNα/β signaling. Several viral proteins, such as the influenza virus NS1 protein and the human papilloma virus (HPV) E6 oncoprotein inhibit expression of type I IFN by blocking the activation or activity of interferon regulatory factor 3 (IRF3), a transcription factor important for type I IFN production (Ronco et al., 1998; Talon et al., 2000). The vaccinia virus protein B18R is secreted from cells and binds IFN in the extracellular space to prevent its binding to cells (Alcamí and Smith, 1995; Colamonti et al., 1995). Other viral proteins, such as cytomegalovirus (CMV) IE1, measles V protein, and dengue virus NS4B, inhibit the signaling pathway itself (Gao et al., 1997; Muñoz-Jordán et al., 2003; Paulus, Krauss, and Nevels, 2006; Yokota et al., 2003).

Herpes simplex virus 1 (HSV-1) is a large, double-stranded DNA virus that productively infects epithelial cells and establishes a latent infection in sensory ganglia for the life of the host (Roizman, Knipe, and Whitley, 2007). In cells that have been exposed to IFN before infection, HSV-1 replication is severely reduced compared with cells infected in the absence of IFN (Altinkılık and Brandner, 1988; Mittnacht et al., 1988; Oberman and Panet, 1988; Pierce et al., 2005). However, cells that are infected with HSV-1 and then treated with IFN show reduced IFN signaling and decreased ISRE reporter...
gene activity (Chee and Roizman, 2004; Johnson, Song, and Knipe, 2008; Yokota et al., 2001). One anti-IFN activity that has been characterized for HSV-1 is the ICP0-dependent inhibition of IRF-3 stimulated IFNβ expression (Melroe et al., 2007). Second, the HSV-1 late protein γ34.5 binds protein phosphatase 1 to counteract the activity of PKR, by causing the dephosphorylation and reactivation of eIF2α (Chou et al., 1995; He, Gross, and Roizman, 1997, 1998; Leib et al., 2000). We have also shown that HSV-1 ICP27 is necessary and sufficient to inhibit IFNα-induced Stat-1 phosphorylation and nuclear accumulation (Johnson, Song, and Knipe, 2008). The effect was observed by 2–4 hpi, so this is likely an early event in HSV infection.

ICP27 is a multifunctional immediate early protein with homologs in all herpesviruses (Roizman, Knipe, and Whiteley, 2007) that is essential for transcription of some early and late viral proteins (Jean et al., 2001). Early in infection it is mostly nuclear but has been shown to shuttle between the nucleus and cytoplasm later in infection (Clements et al., 2004; Soliman, Sandri-Goldin, and Silverstein, 1997). It has roles in transcriptional regulation through association with RNA polymerase II (Zhou and Knipe, 2002) and translation through association with translation factors eIF3, eIF4g, and PABP (Ellison et al., 2005; Fontaine-Rodriguez and Knipe, 2008; Fontaine-Rodriguez et al., 2004). ICP27 also affects RNA processing through interactions with splicing machinery (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Phelan et al., 1993; Sandri-Goldin and Hibbard, 1996) and regulation of differential polyadenylation (Hann et al., 1998; McGregor et al., 1996; McLaughlan et al., 1992; McLaughlan, Simpson, and Clements, 1989). ICP27 associates with RNA via its RGG box and CR1 regions to stabilize A/U-rich RNAs (Brown et al., 1995; Ingram et al., 1996). In some studies, it has also been implicated in nuclear export of some viral transcripts (Koffa et al., 2001; Mears and Rice, 1998; Pearson, Knipe, and Coen, 2004; Sandri-Goldin, 1998; Wadd et al., 1999). However, other groups have not seen a difference in RNA export during infection with ICP27 mutant viruses (Ellison et al., 2005; Fontaine-Rodriguez and Knipe, 2008; Pearson, Knipe, and Coen, 2004).

We performed immunofluorescence experiments to determine that ICP27 was necessary and sufficient for inhibition of Stat-1 phosphorylation and nuclear accumulation. In these experiments we also observed that even after IFNα-treatment, many cells that did not stain positive for ICP27 still did not show nuclear accumulation of Stat-1 (Johnson, Song, and Knipe, 2008). It appeared that ICP27 expression was causing a bystander effect in surrounding cells through an unknown mechanism.

There have been several hypotheses about the mechanism(s) by which IFN signaling is inhibited by HSV-1, with possible mechanisms being the HSV-1 virion host shut-off protein (vhs) or the cellular suppressor of cytokine signaling protein SOCS-3 (Chee and Roizman, 2004; Yokota et al., 2005, Yokota et al., 2004). However, the actual mechanism of inhibition is still unknown. In this study, we show that HSV-1 infection inhibits IFN signaling at or before the phosphorylation of Jak-1. In exploring the bystander effect of ICP27 on surrounding cells further, we have also found that HSV-1 infection and ICP27 transfection cause the secretion of a heat-stable, protease-sensitive soluble factor that inhibits IFNα-induced Stat-1 nuclear accumulation in trans.

Results

HSV-1 infection causes bystander cell inhibition of IFNα-induced Stat-1 nuclear accumulation

In our previous study, we observed that a number of cells that did not stain for ICP27 showed mostly cytoplasmic distribution of Stat-1 even after treatment with IFNα (Johnson, Song, and Knipe, 2008). To examine the relationship between ICP27 and Stat-1 distribution in HSV-1 infected cells, we mock-infected or infected Vero cells with WT HSV-1 for 10 h and treated with IFNα at 10^4 U/ml for 30 min prior to fixation. Cells were stained with antibodies to Stat-1 and ICP27 and 200–250 cells per cover slip were scored blindly for Stat-1 localization, as being nuclear, cytoplasmic, or both (Fig. 1A, black arrow–cytoplasmic, white arrow–nuclear, white arrow head–both).

In the absence of IFN, Stat-1 was mostly cytoplasmic in over 70% and both nuclear and cytoplasmic in over 25% of mock-infected cells, but after IFN treatment Stat-1 was redistributed to be approximately 75% nuclear and about 25% cytoplasmic and nuclear (Fig. 1B). After HSV-1 infection, when roughly 20% of cells appeared to be infected (as detected by ICP27 immunofluorescence), Stat-1 was cytoplasmic in nearly 70% of cells and both cytoplasmic and nuclear in about 30% of cells in the absence of IFN (Fig. 1B). After IFNα treatment however, Stat-1 accumulated in the nucleus of only approximately 45% of cells, which is significantly lower than in mock-infected cells (p<0.01).

![Fig. 1](https://example.com/image1.png)

**Fig. 1.** HSV-1 infection inhibits IFNα-induced nuclear accumulation of Stat-1 in surrounding cells. Vero cells were mock infected or infected (MOI = 3) with WT HSV-1 for 10 h and treated with IFN for 30 min before fixation, as indicated. Immunofluorescence was done with antibodies towards Stat-1 and ICP27. Stat-1 localization was scored as being predominantly nuclear (A: white arrow), predominantly cytoplasmic (A: black arrow), or both (A: white arrow head). The percent of cells infected was determined by counting cells that stained positive for ICP27 (B). Data shown are from cell counts from replicate cover slips, and statistical analysis was performed with the Student’s t-test. The experiment shown is representative of multiple experiments.
Consistent with previous results, cells that were transfected with empty vector showed predominantly cytoplasmic staining for Stat-1 (90% of cells, Fig. 2). After treatment with IFNα, over 85% of empty vector-transfected cells showed Stat-1 accumulation in the nucleus (Fig. 2). Cells that were transfected with an ICP27 expression vector plasmid showed an even more dramatic effect than cells infected with WT HSV-1, with nearly 100% cytoplasmic Stat-1 localization in the absence of IFN and only approximately 1% of cells with nuclear staining after IFN treatment (p<0.001, Fig. 2). About 15% of cells stained positive for ICP27 (data not shown). These data suggested that ICP27 expression is sufficient to affect the type I IFN signaling in surrounding cells, although it was possible that the other cells expressed ICP27 at levels below the detection threshold of our antibody.

ICP27 causes the release of a heat-stable, protease-sensitive, IFN-antagonizing factor

Some large DNA viruses encode proteins that are secreted from cells and compete for binding with IFN and IFNAR (Alcamí and Smith, 1995; Colamonici et al., 1995). To determine if the inhibition of Stat-1 nuclear accumulation that we observed was the result of a factor secreted by ICP27-expressing cells, we harvested medium from cells transfected with an empty vector or pCI-ICP27, transferred the medium to new cells and then treated the cells with IFNα, and scored for Stat-1 localization as above. We observed that cells incubated in medium from cells transfected with pCI plasmid had predominantly cytoplasmic Stat-1 in the absence of IFNα (69%, Table 1), and that after IFNα treatment, Stat-1 accumulated in the nucleus in most cells (81%, Table 1). Cells grown in medium from cells transfected with the ICP27 plasmid also showed predominantly cytoplasmic Stat-1 in the absence of IFN (81%, Table 1), but the IFN-induced accumulation of Stat-1 in the nucleus occurred in a significantly smaller percentage of cells (37%) than in cells grown in medium from pCI-transfected cells (p<0.05, Table 1). Therefore, ICP27 expression caused the secretion of a soluble IFN-antagonizing factor.

Table 1
ICP27 causes the secretion of a heat-stable protease-sensitive IFN antagonist that is 10–50 kDa.

<table>
<thead>
<tr>
<th>Medium from cells transfected with</th>
<th>Treatment</th>
<th>IFN</th>
<th>% cells with Stat1 localization*</th>
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<tr>
<td></td>
<td></td>
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<td>Nucleus</td>
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<tr>
<td>Empty vector</td>
<td>None</td>
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<td>7 ± 1</td>
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<td>ICP27 vector</td>
<td></td>
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<td>81 ± 5</td>
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<td></td>
<td>1 ± 1</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>37 ± 19, p&lt;0.05+</td>
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<tr>
<td>Empty vector</td>
<td>55 °C 1 h, 95 °C 10 min</td>
<td></td>
<td>0.5 ± 0.5</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>75 ± 4</td>
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<td>2 ± 2</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>41 ± 3, p&lt;0.01+</td>
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<tr>
<td>Empty vector</td>
<td>55 °C 1 h, 95 °C 10 min with proteinase K</td>
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<td>0.5 ± 0.5</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>58 ± 7</td>
</tr>
<tr>
<td>ICP27 vector</td>
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<td></td>
<td>0.5 ± 0.5</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>72 ± 1, p&lt;0.05</td>
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<tr>
<td>Empty vector</td>
<td>Filtered through 50 kDa pores</td>
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<td>1 ± 0.5</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>84 ± 2</td>
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<tr>
<td>ICP27 vector</td>
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<td>2 ± 1</td>
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<td>59 ± 3, p&lt;0.05+</td>
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<td>Empty vector</td>
<td>Filtered through 10 kDa pores</td>
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<td>3 ± 1</td>
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<td>3 ± 1</td>
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<td></td>
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<td>77 ± 4, p&lt;0.25</td>
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</table>

* Vero cells were transfected with empty vector (pCI) or an ICP27 expression vector (pCI-ICP27). At 24 h post-transfection, medium from each culture was harvested and left untreated, heated to 55 °C for 60 min and to 95 °C for 10 min in the absence or presence of proteinase K, or passed through a 50- or 10-kDa filter before being transferred to naïve Vero cells. At 24 h after media transfer, these cells were treated with IFNα at 104 U/mL for 30 min, as indicated, fixed, and stained for Stat-1. The distribution of Stat-1 localization was determined as in Fig. 1.

+ Compared with control medium from cells transfected with empty vector.
To characterize the ICP27-induced secreted factor, we assayed its heat stability. Vero cells were transfected with empty vector or ICP27 expression vector. At 24 h post-transfection, we harvested the medium from the transfected cells and incubated it at 55 °C for 60 min, at 95 °C for 10 min, cooled it to 37 °C, and transferred the medium to new Vero cells. These cells were then treated with IFNβ for 30 min, fixed, stained with antibodies to Stat-1, and scored for Stat-1 localization as above.

Consistent with previous results, cells incubated in medium from empty vector- or ICP27 expression plasmid-transfected cells showed mostly cytoplasmic Stat-1 localization in the absence of IFN (68% and 75%, respectively, Table 1). However, after IFNβ treatment, Stat-1 was nuclear in 75% of cells grown in medium from empty vector-transfected cells, but in only 41% of cells grown in medium from ICP27 plasmid-transfected cells (Table 1), significantly different ($p<0.01$) from cells treated with control medium.

To further determine the molecular nature of the secreted factor, we incubated the media with proteinase K, and transferred them to new Vero cells, which were treated with IFNβ as indicated (Table 1) for 30 min, fixed, stained, and scored for Stat-1 localization. Cells grown in proteinase K-treated medium from cells transfected with empty vector or ICP27 expression vector showed mostly cytoplasmic Stat-1 localization in the absence of IFN treatment (82% and 79% of cells, respectively, Table 1). Cells grown in proteinase K-treated medium had mostly nuclear localization after IFNβ treatment, regardless of whether the source of the medium was cells transfected with empty vector or ICP27 expression vector (58% and 72%, respectively, Table 1).

To determine the approximate size of the factor released from cells that express ICP27, we transfected cells with empty vector or ICP27 expression plasmid, harvested the medium as above, and passed it through molecular sizing filters with 10 or 50 kDa molecular weight thresholds before overlaying naïve Vero cells. Cells were treated with IFNβ, stained, and scored for Stat-1 localization as above.

In the absence of IFNβ, cells that were grown in medium from cells transfected with either plasmid filtered through the 50-kDa filter showed mostly cytoplasmic Stat-1 localization (70%, Table 1). After IFNβ treatment, there was a shift to mostly nuclear Stat-1 in cells grown in medium from pCI-transfected cells (84%, Table 1), which was significantly decreased in cells grown in medium from pCI-ICP27-transfected cells (59%, $p<0.005$, Table 1). Cells grown in medium passed through the 10-kDa filter showed mostly cytoplasmic Stat-1 localization in the absence of IFN (70% for medium from pCI-transfected cells, 67% for medium from pCI-ICP27-transfected cells, Table 1). After IFNβ treatment, Stat-1 accumulated in the nucleus of most cells grown in medium from pCI-transfected cells (74%, Table 1) and cells grown in medium from pCI-ICP27-transfected cells (77%, Table 1).

In total, these results argued that the IFN-antagonizing factor secreted from ICP27-expressing cells was a heat-stable, protease-sensitive protein between 10 and 50 kDa in molecular weight.

Stage of IFN signaling pathway affected by HSV

Type I, but not type II, IFN-induced Stat-1 phosphorylation is inhibited by HSV-1 infection

We have shown that ICP27 expression causes the secretion of a soluble protein that inhibits IFNβ-induced Stat-1 nuclear localization. However, the stage of the signaling pathway affected was still unknown. Jak-1 and Stat-1 are shared factors between the type I and type II IFN signaling pathways (Samuel, 2001). Type II IFN signaling has been shown to be affected by HSV-1, as detected by reporter gene assay, albeit at significantly reduced levels compared with the inhibition of type I IFN signaling (Yokota et al., 2001). To see if type II IFN-dependent Stat-1 phosphorylation was inhibited under our infection conditions, we mock infected or infected Vero cells for 10 h and treated them with IFNγ or IFNβ at $10^5$ U/mL as indicated, before Western blot analysis with antibodies for pStat-1, Stat-1, ICP27, and actin. Stat-1 was phosphorylated in mock-infected cells following treatment with either type of IFN (Fig. 3, lanes 2 and 3). However, in HSV-1 infected cells, Stat-1 was phosphorylated only after treatment with IFNγ (Fig. 3, lane 6), not with IFNβ (Fig. 3, lane 5). These results argued that HSV-1 affects type I but not type II signaling. It is therefore very likely that the inhibition occurs at or before Jak-1 activation.

HSV-1 infection inhibits IFNβ-induced ISG15 expression in an ICP27-independent manner

Other studies have shown that HSV-1 inhibited reporter gene activity from constructs containing ISREs and GASs (Yokota et al., 2001). However, there were no published reports of the effects of ICP27 on the expression of endogenous ISG expression. We therefore tested the capacity of HSV-1 to inhibit expression of the ISG15 protein, a ubiquitin-like interferon-stimulated protein that gets conjugated to other proteins with as yet undetermined consequences (Biron and Sen, 2007; Zhao et al., 2004, 2005).

We mock-infected or infected Vero cells with WT HSV-1 or ICP27 virus at an MOI of 20 for 10 h and treated with IFNβ 2 h before harvest, as indicated (Fig. 4). Western blot analysis was performed with antibodies specific for pStat-1, ISG15, and GAPDH. Phosphorylation of Stat-1 and ISG15 expression were induced in mock-infected cells after treatment with IFNβ (Fig. 4, lane 2). Consistent with previous results, cells infected with WT HSV-1, IFNβ treatment showed reduced Stat-1

![Fig. 3](image-url)  
Fig. 3. Type I, but not type II, IFN-induced Stat-1 phosphorylation is inhibited by HSV-1. Vero cells were mock infected (lanes 1–3) or infected (MOI = 20) with WT HSV-1 (KOS, lanes 4–6) and treated with IFN (lanes 2, 3) or IFN (lanes 3, 6) for 30 min before harvest. Western blot analysis was done with antibodies specific for pStat-1, Stat-1, ICP27, and actin.

![Fig. 4](image-url)  
Fig. 4. HSV-1 inhibits IFN-induced ISG15 expression in an ICP27-independent manner. Vero cells were mock infected (lanes 1–2) or infected (MOI = 20) with WT HSV-1 (KOS, lanes 3–4), or ICP27 virus (3d1,2, lanes 5–6) and treated with IFN (even lanes) for 2 h before harvest. Western blot analysis was done with antibodies specific for pStat-1, ISG15, and GAPDH.
phosphorylation and no evidence of ISG15 expression (Fig. 4, lane 4). Surprisingly, though Stat-1 was phosphorylated after IFNα treatment in cells infected with the ICP27− virus, ISG15 expression was not induced (Fig. 4, lane 6). These data suggested that there are ICP27-dependent and ICP27-independent mechanisms of inhibition of type I IFN signaling.

**HSV-1 infection does not alter levels of Interferon α/β receptor chain 1 or 2 (IFNAR1, 2)**

Because HSV-1 infection inhibited type I, but not type II IFN signaling, we reasoned that a signaling activity upstream of Jak-1 such as the type I IFN receptor might be affected. Previous reports with vhs mutant viruses showed that late in infection there was a slight decrease in the protein levels for the IFNα/β receptor (Chee and Roizman, 2004). However, we and others have observed an effect of HSV-1 on IFN signaling at much earlier times post-infection than the time when IFN receptor levels decreased (Johnson, Song, and Knipe, 2008; Yokota et al., 2001).

To determine if there was a decrease in IFNAR levels in our infection conditions, Vero cells were mock-infected or infected with WT HSV-1 (KOS) at an MOI of 20 for 2 or 10 h and treated with interferon 30 min before harvest. Western blot analysis was done with antibodies specific for IFNAR1, IFNAR2, and GAPDH. There were no consistent changes in IFNAR1 or IFNAR2 levels, regardless of infection or treatment with IFN (Fig. 5). These results suggested that any degradation of IFNAR1 and IFNAR2 at later times post-infection is not the major mechanism of the inhibition of type I IFN signaling.

**HSV-1 infection does not cause differential splicing or nuclear export of IFNAR2 RNA**

We showed above that ICP27 is sufficient to cause release of a soluble heat-stable peptide that inhibits type I IFN signaling. Because ICP27 has been implicated in RNA processing and nuclear export (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Pearson, Knipe, and Coen, 2004; Phelan et al., 1993; Sandri-Goldin and Hibbard, 1996; Scibica, Dai, and Sandri-Goldin, 2003; Wadd et al., 1999) and has been shown to stabilize IFNβ RNA (Mosca, Pitha, and Hayward, 1992), we hypothesized that ICP27 might affect splicing of the transcript from the IFNAR2 gene. The mRNA for IFNAR2, the major IFN-binding subunit of the receptor (Novick, Cohen, and Rubinstein, 1994), has a splicing variant that encodes a 27 kDa secreted form of the receptor (Hardy et al., 2001; Lutfalla et al., 1995; Novick, Cohen, and Rubinstein, 1994). This secreted IFNAR2 has been shown to inhibit type I IFN signaling in cells that express full-length IFNAR2 (Hardy et al., 2001). Therefore, ICP27 might be affecting the splicing or nuclear export of IFNAR2 RNAs and causing preferential expression of the secreted IFNAR2 protein.

To determine if ICP27 was affecting the splicing of IFNAR2 mRNAs, we mock infected or infected Vero cells with WT or ICP27− HSV-1, and at 10 h, we isolated RNA and subjected it to Northern blot analysis with a hybridization probe specific for a region of the IFNAR2 transcript that is shared between splice variants (Fig. 6A). There was no apparent difference in the ratio of IFNAR2 RNA splice variant levels between mock, WT, and ICP27−-infected cells (Fig. 6A).

Although there was no difference in the whole cell IFNAR2 mRNA levels, we hypothesized that ICP27 might be causing differential nuclear export of the IFNAR2 mRNA splice variants. Vero cells were mock-infected or infected with WT or ICP27− HSV-1 for 10 h, and RNA was isolated from nuclear and cytoplasmic fractions. Northern blot analysis was performed as above with probes specific for IFNAR2 mRNA and U3 snRNA, the latter as a control for our fractionation. There was no apparent difference in relative nuclear to cytoplasmic levels of the sIFNAR2 vs. IFNAR2 RNA species between mock, WT, or ICP27−-infected cells (Fig. 6B). These results argued that the mechanism by which ICP27 induces the secreted inhibitory factor did not involve splicing or nuclear export of IFNAR2 mRNAs.

**Discussion**

Type I IFN is one of the first lines of defense that a host mounts against viral infection. As such, it is important for the fitness of many viruses that they evade the induction or the effects of type I IFN. Several viruses do this by antagonizing either the activation or activity of IRF-3 to prevent type I IFN expression (Ronco et al., 1998; Talon et al., 2000). In contrast, other viruses inhibit the IFN signaling pathway itself through interactions with and/or degradation of the Jak/Stat factors (Basler et al., 2000; Gao et al., 1997). Thus far, the only virus family found to encode a secreted type I IFN antagonist is *Poxviridae*. Vaccinia, tanapox, and ectromelia viruses encode secreted IFNα/β receptor homologs that inhibit IFN signaling.

HSV-1 encodes several IFN antagonists, including ICP0, which inhibits IRF-3 nuclear accumulation (Melroe, DeLuca, and Knipe, 2004); ICP27, which inhibits IFNα-induced Stat-1 phosphorylation and nuclear accumulation (Johnson, Song, and Knipe, 2008), and γ34.5, which counteracts the activity of PKR (Chou et al., 1995; He, Gross, and Roizman, 1997; Johnson, Song, and Knipe, 2008; Leib et al., 2000), an ISG that phosphorylates the translation initiation factor eIF2α to inhibit protein synthesis (He, Gross, and Roizman, 1998; Samuel, 1979a,b).

**Fig. 5.** Type I IFN receptor levels do not change over the course of infection. Vero cells were mock infected (lanes 1–4) or infected (MOI = 20) with WT HSV-1 (KOS, lanes 5–8) for 2 h (odd lanes) or 10 h (even lanes) and treated with IFN for 30 min before harvest (lanes 3–4, 7–8). Western blot analysis was done with antibodies specific for IFNAR1, IFNAR2, and GAPDH.

**Fig. 6.** ICP27 does not affect the splicing or nuclear export of IFNAR2 transcripts. Vero cells were mock-infected or infected (MOI = 20) with wt HSV-1 (KOS, A: lane 2, B: lanes 3–4) or ICP27− virus (5dl1.2, A: lane 3, B: lanes 5–6) for 10 h. RNA was harvested from whole cells (A) or cells fractionated into nuclear and cytoplasmic components (B) and Northern blot analysis was done with probes specific for IFNAR2 and U3 snRNA.
HSV-1 infection or ICP27 expression causes the inhibition of type I IFN
signaling in surrounding cells

We observed that HSV-1 infection at a fairly low MOI causes
inhibition of Stat-1 nuclear accumulation in a larger percentage of
cells than are infected, as determined by ICP27 staining. We also show
that transfection of cells with a plasmid encoding ICP27 is sufficient
for the inhibition of Stat-1 nuclear accumulation in a larger percentage of
cells than stain positive for ICP27. This indicates that
either ICP27 is present at levels below detection by immunofluores-
cence in all cells in which we see no nuclear accumulation of Stat-1, or
that HSV-1 infection and ICP27 expression cause the secretion of an
inhibitory factor that affects the surrounding cells, possibly similar in
mechanism to the vaccinia virus B18R protein (Alcamí and Smith,
1995; Colomonic et al., 1995). This effect could have very important
implications for viral spread because HSV-1 replication is inhibited in
cells that have been pre-exposed to IFNαx (Altinkılık and Brandner,
1988; Mittnacht et al., 1988).

ICP27 expression causes the release of a heat-stable, protease-sensitive soluble IFNαx antagonist

To differentiate between the two models described above, we
transferred medium from cells transfected with empty vector or an
ICP27 expression vector to naive Vero cells. We found that medium
harvested from cells transfected with empty vector has no effect on
IFNα-induced nuclear accumulation of Stat-1. However, there is
inhibition of Stat-1 nuclear accumulation conferred by medium from
cells transfected with an ICP27 expression plasmid. We also found
that this inhibitory activity is heat-stable and protease sensitive. These
results suggested that there is a stable peptide secreted from ICP27-
expressing cells that affects the IFN signaling in surrounding cells.

These results also raise the possibility that the secreted factor is
either a cellular protein or all or part of ICP27. We were unable to
detect ICP27 in the overlaid cells by immunofluorescence or in the
medium by Western blot (data not shown). Because the commercially
available antibodies to ICP27 are all to N-terminal epitopes, however,
it is possible that there is a C-terminal cleavage product of ICP27 that
causes the inhibition.

HSV-1 infection inhibits type I but not type II IFN-dependent Stat-1 phosphorylation

Jak-1 and Stat-1 are involved in both type I and type II IFN
signaling. We and others have shown that HSV-1 inhibits IFNα-
induced Stat-1 phosphorylation (Chee and Roizman, 2004; Mittnacht
et al., 1988; Yokota et al., 2001). To determine if Stat-1 or Jak-1 were
specifically targeted by the virus, we tested the capacity of HSV-1 to
inhibit IFNα-induced Stat-1 phosphorylation as well, and we found that
IFNα-induced Stat-1 phosphorylation is not inhibited by HSV-1.
Because Jak-1 activation is necessary for Stat-1 phosphorylation (Darnell,
Kerr, and Stark, 1994; David et al., 1993; Johnson, Song, and
Knipe, 2008; Plataniastis, Uddin, and Colomonic, 1994) these results
suggested that the effect of HSV-1 was on a factor or signaling event
upstream of Jak-1 activation. This could include an effect on IFNαx,
itself, one or both of the receptor proteins, or the interactions between
the receptor and the janus kinases.

The lack of inhibition of IFNα-induced Stat-1 phosphorylation
contrasts somewhat with previous studies that show a decrease in
IFNα-induced luciferase activity from an ISRE reporter construct and a
less pronounced decrease in IFNα-induced luciferase from a GAS
reporter construct. However, we have also shown that though HSV-1
inhibition of IFNα-induced Stat-1 phosphorylation and nuclear
accumulation is ICP27-dependent, there is ICP27-independent inhi-
bitiion of IFNα-induced ISG15 expression. This suggested the
possibility of multiple levels of inhibition of the type I IFN signaling
pathway. Another immediate early protein, ICPO has been shown to be
important for the inhibition of expression of some ISGs, including
ISG54, ISG56, and ISG15 possibly through its activity as a ubiquitin
ligase (Eidson et al., 2002). It is also possible that HSV-1 encodes an
activity that inhibits the association of Stat-1 with DNA or with
important transcription co-factors, such as CBP/p300. This would
result in the inhibition of type I and type II IFN-induced gene
expression even if Stat-1 phosphorylation is not affected.

HSV-1 infection has no effect on the levels of the type I IFN
receptor subunits

Because of the implications that the step of IFNαx signaling that is
affected by HSV-1 is upstream of Jak-1 activation, we looked at the
stability of the type I IFN receptor. In our hands, both IFNAR1 and
IFNAR2 levels are stable in cells up to 10 hpi. This argues that HSV-1 is
not causing the degradation of IFNAR in time to effect the inhibition of
IFNα-dependent Stat-1 phosphorylation. A previous study (Chee
and Roizman, 2004) showed a decrease in the levels of one of the
receptor proteins, beginning at 8 hpi; however, they infected HeLa
cells with HSV-1 strain F, which may cause more protein degradation
than the KOS strain. It is possible that HSV-1 strain KOS does cause
degradation of IFNAR at later times post-infection. However, it is also
possible that the decrease in IFNAR levels seen in that study is due
to normal degradation of the protein but decreased synthesis of
IFNAR due to the host protein synthesis shut-off functions of vhs and
ICP27.

Although the type I receptor levels appear constant through the
time post-infection that IFNαx-induced Stat-1 phosphorylation is
inhibited, it is possible that the subcellular localization of the protein
is altered by HSV-1 infection. If, for example, one or both of the
receptor subunits were internalized during HSV-1 infection, there
would be decreased sensitivity of infected cells to IFNαx treatment.
HSV-1 has previously been shown to cause internalization of the EGF
receptor via interactions between the HSV-1 protein ICPO and the
cellular proteins CIN85 and Cbl (Liang, Kurakin, and Roizman,
2005).

HSV-1 does not affect the splicing or nuclear export of IFNAR2 mRNAs

ICP27 has several functions, including inhibition of splicing,
causing differential use of polyadenylation signals, and roles in RNA
export from the nucleus (Hann et al., 1998; Hardwicke and Sandri-
Goldin, 1994; Hardy and Sandri-Goldin, 1994; Koffa et al., 2001; Liang,
Kurakin, and Roizman, 2005; McGregor et al., 1996; McLauchlan et al.,
1992; McLauchlan, Simpson, and Clements, 1989; Phelan et al., 1993;
Sciabica, Dai, and Sandri-Goldin, 2001; Wadd et al., 1999). ICP27 has
recently also been shown to promote production of a secreted form of
the HSV-1 glycoprotein gC by causing the transmembrane domain
coding sequences to be spliced out of the mRNA (Sedlackova et al.,
2008).

The mRNA for IFNAR2 has four known splice variants encoding
three proteins with some shared domains (Lutfalla et al., 1995): the
full-length signaling molecule, a dominant-negative molecule con-
taining the IFN-binding extracellular and transmembrane domains
(de Weerd, Samaraijiwa, and Hertzog, 2007; Gazzioa et al., 2005), and
a secreted form with only the IFN-binding ectodomain, which has
been shown to inhibit signaling in cells with a full complement of
IFNAR2 (de Weerd, Samaraijiwa, and Hertzog, 2007; Lutfalla et al.,
1995; Novick, Cohen, and Rubinstein, 1994). We hypothesized that
ICP27 might affect the splicing or nuclear export of the splice variants
of IFNAR2, favoring the expression of the secreted form of
the receptor. However, we found no differences in the ratios of secreted
IFNAR2 RNA to full-length IFNAR2 RNA between mock-, WT-, and
ICP27−infected cells. We were also unable to find siIFNAR2 by
Western blot in the medium from cells transfected with an ICP27
expression vector (data not shown). These results are certainly
suggesstive that sIFNAR2 is not the secreted factor induced by ICP27 expression that inhibits Stat-1 nuclear accumulation in response to IFNα. However, it is possible that ICP27 causes increased secretion of sIFNAR2 from cells. It is also possible that ICP27 expression causes the expression and secretion of a dominant negative IFN from cells.

These results have very important implications for HSV-1 spread and evasion of innate immunodetection because HSV-1 replication is inhibited in cells that have been exposed to IFN before infection (Hardy et al., 2001; Mittnacht et al., 1988; Oberman and Panet, 1988). This inhibition makes it very difficult for the virus to replicate efficiently, infect new cells, and establish latency in the host. By causing the secretion of a factor that inhibits IFNα signaling in neighboring, uninfected cells, HSV-1 maintains a cellular environment conducive to its own replication and spread. The inhibition of the signaling pathway upstream of Jak-1 activation may be due to the secreted protein competing for binding between IFNα and IFNAR2. This would be consistent with our data showing no difference in IFNAR levels after 10 h of infection and the inhibition of type I but not type II IFN-induced Stat-1 phosphorylation.

Materials and methods

Cells and viruses

Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL) supplemented with 5% heat-inactivated fetal calf serum (FCS) and 5% heat-inactivated newborn calf serum (BCS).

Viral stocks were grown in the appropriate complementing cell lines (the HSV-1 WT KOS was grown on Vero cells and 5dI1.2 (McCarthy, McMahen, and Schaffer, 1989) was grown on V827 cells. Viral titers were determined by plaque assay in Vero cells or the indicated complementing cell line as described (Knipe and Speng, 1982). For infection experiments, viruses were used at an MOI of 1 or 2 to infect Vero cells, which were incubated at 37°C in PBS with 1% BCS, 0.1% glucose for 1 h before removal of the inoculum and addition of DMEM supplemented with 1% FCS.

Western blots

Mock-infected or infected cells were harvested from 25-cm² flasks in 400 µL of SDS sample buffer (62.5 mM Tris–HCl pH 6.8, 20% glycerol, 2% SDS, 0.1% bromophenol blue, 10 mM β-glycerophosphate, 5 mM sodium fluoride, 1 mM sodium vanadate, 0.5% β-mercaptoethanol) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% bis-crosslinked polyacrylamide gels and electrically transferred to nitrocellulose in transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol) overnight at 4°C with the Bio-Rad Transblot system. Membranes were probed with antibodies to Stat-1 (1:1000), pStat-1 (1:500), actin (1:1000), IFNAR1 (1:750), or IFNAR2 (1:500) from Santa Cruz Biotechnology, Inc., ICP27 (H1119 at 1:10,000) from Virusys, and GAPDH (1:40,000) from Abcam, in PBST (0.5% Tween 20 in PBS from Gibco), washed twice for 5 min in PBST, and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) diluted 1:5000–1:20,000 from Santa Cruz Biotechnology, Inc. HRP activity was detected using Western Lightening chemiluminescence reagent (PerkinElmer) or Lumilight Western blotting substrate (Roche) and exposed on X-ray film (Kodak).

Plasmids and transfections

Plasmid pCI was obtained from Promega. Plasmid pCI-ICP27 has been described previously (Olesky et al., 2005). At 24 h before transfection, Vero cells were seeded into six-well plates containing glass coverslips for fluorescence experiments. Transfections were carried out using Opti-MEM medium (Gibco) and Genejuice reagent (Novagen) according to the manufacturer’s instructions.

Immunofluorescence

Vero cells were seeded for immunofluorescence at 5 × 10⁵ cells/ well on glass coverslips in six-well plates and incubated overnight at 37°C before infection or transfection, after which they were treated as indicated with IFNα (PBL biomedical laboratories). Following incubation for the appropriate time, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, and cells were permeabilized by incubation in methanol at −20°C for 2 min. Following several washes in PBS, cells were incubated overnight in IF buffer (PBS containing 2.5% goat serum [Sigma]). Dylight-488 conjugated streptavidin (Pierce) or primary antibodies were diluted appropriately (Streptavidin-488 1:50, STAT-1 1:50) and applied to cells in PBS, and the cells were incubated for 45 min at 37°C. Cells were washed twice for 5 min in PBS. Secondary antibodies conjugated to Alexa 594 and Alexa 488 dyes were obtained from Molecular Probes, Inc., and were applied at 1:1000 in PBS for 30 min at 37°C. Cells were then washed twice for 10 min in PBS at room temperature, and coverslips were mounted with Prolong antifade reagent (Molecular Probes, Inc.).

For cell culture medium transfer experiments, medium was removed from transfected Vero cells at 24 h post-transfection, treated as indicated by heating to 55°C for 60 min and then to 95°C for 10 min in the presence or absence of 100µg/mL proteinase K (Roche), and cooled to 37°C or spun through 10 or 50 kDa molecular weight cut off Microcon® filters (Millipore) according to the manufacturer’s instructions before overlaying new Vero cells on coverslips.

Slides were viewed with an Axiosplan 2 microscope (Zeiss) with a 63× objective and a 10× ocular objective. Images were collected with the Axiosvision 4.5 suite of programs (Zeiss) and a Hamamatsu C4742–95–12NR digital camera.

RNA isolation and Northern blot analysis

Total RNA was isolated from Vero cells using TRI reagent (Ambion) according to the manufacturer’s instructions. To separate RNA into nuclear and cytoplasmic fractions, cells were lysed by incubation for 5 min on ice in lysis buffer (50 mM Tris–Cl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.5% v/v Nonidet P-40, filter sterilized) and nuclei were removed by centrifugation. RNA was isolated from nuclei using TRI reagent, according to the manufacturer’s instructions. RNA was isolated from the cytoplasm by adding SDS to 0.05% and extracting twice with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. Cytoplasmic RNA was ethanol precipitated and resuspended in H₂O.

For Northern blots, 5ug of RNA of each sample was resolved in an agarose gel using the NorthenMax–Gly™ kit (Ambion) and transferred to BrightStar® Plus positively charged nylon membrane (Ambion) according to the manufacturer’s instructions. DNA probes for IFNAR2 (5′–GCTACATCACTGCTCTTAAAACAGATACAC GTAGTTCTGTTT GGAA–3′) and U3 (5′–ACCACTAGACGCGTTCCTCCCT CTACCCC– CAATACGAGAAGAACG–3′) were obtained from IDT and biotinylated using BrightStar® Psoralen-biotin nonisotopic labeling kit (Ambion) according to the manufacturer’s instructions. Probes were hybridized using UltraHyb® hybridization buffer (Ambion) and blots were washed and RNA detected using the BrightStar® biodetect kit (Ambion) according to the manufacturer’s instructions.

References

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