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Effects of interferon on immediate early mRNA and protein levels in sensory neuronal cells infected with herpes simplex virus type 1 or pseudorabies virus

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15

Abstract

Most alphaherpesviruses are able to establish latency in sensory neurons and reactivate upon specific stimuli to cause recurrent symptoms. We have previously shown that interferon (IFN) is capable of inducing a quiescent HSV-1 and PRV infection that strongly resembles *in vivo* latency in primary cultures of TG neurons. This IFN-induced latency-like quiescence was
20 found to correlate with suppression of the immediate early protein ICP4 in HSV-1 and its ortholog IE180 in PRV. Here, we mechanistically investigated the IFN-mediated suppression of ICP4 and IE180 in sensory neuronal cells. Using RT-qPCR, mRNA levels of either HSV

ICP4 or PRV IE180 at 4hpi were mildly but not significantly different in IFN-treated samples
25 versus control samples, whereas a strong reduction was observed at 8hpi and 12hpi. However,
at 4hpi, HSV ICP4 but not PRV IE180 protein expression was already markedly reduced in
IFN-treated samples. In line with this difference in IFN-mediated suppression of HSV ICP4
versus PRV IE180 protein levels, we found that IFN resulted in an increase in
phosphorylation of the translation initiation factor eIF2 α in HSV-infected but not in PRV-
30 infected cells. The latter finding indicates that PRV efficiently circumvents IFN-mediated
translation inhibition by interfering with phosphorylation of eIF2 α .

Keywords: alphaherpesvirus, interferon, gene regulation, translational control

1. Introduction

The human herpes simplex virus type 1 (HSV-1) and the porcine pseudorabies virus (PRV) are closely related and belong to the alphaherpesvirus subfamily of the *Herpesviridae*.

A central characteristic of most alphaherpesviruses is their ability to establish a lifelong latent infection in sensory neurons. During HSV-1 and PRV latency, no infectious virus or viral proteins are produced (Roizman and Knipe, 2001). Specific stimuli, often associated with stress and/or immune suppression, can lead to reactivation of the virus from latency, which may be associated with recurrent virus spread and disease symptoms, such as cold sores with HSV-1 (Sainz et al., 2001). The establishment and control of latency and the induction of reactivation are regulated by an incompletely understood interplay between the virus, the neuron and several components of the immune system (Decman et al., 2005).

The interferon (IFN)-mediated innate immunity is at the front line of host defence against viral infections. IFNs are able to interfere with several steps of virus replication. One important example is the IFN-mediated shutdown of mRNA translation through phosphorylation and thereby inactivation of the translation initiation factor eIF2 α and through activation of RNase L (Goodbourn et al., 2000). IFNs have been reported to be very important in limiting replication and spread of alphaherpesviruses (Hendricks et al., 1991; Mikloska and Cunningham, 2001; Sainz and Halford, 2002). Recently, we have demonstrated that IFN is able to drive both HSV-1 and PRV into a latency-like quiescent state in *in vitro* cultures of sensory neurons (De Regge et al., 2010).

Importantly, IFN-mediated suppression of the immediate early (IE) protein ICP4 of HSV-1 or its counterpart IE180 in PRV appeared to be a key step in the establishment of *in vitro* latency and IFN-mediated IE protein suppression was more rapid and efficient in HSV-1 compared to PRV (De Regge et al., 2010).

The aim of the current study was to further mechanistically investigate the suppressive effect of IFN on ICP4 and IE180 levels in sensory neuronal cells. We report that, at the mRNA level, IFN reduced ICP4 and IE180 mRNA expression to a similar extent, in both cases predominantly late in infection. At the protein level, IFN α was found to rapidly and efficiently shut down ICP4 protein expression whereas IE180 protein expression was diminished slower and less efficient, in line with our earlier observations. Remarkably, in HSV-1 infected sensory neuronal cells, IFN caused a rapid and marked phosphorylation and therefore inactivation of the translation initiation factor eIF2 α , whereas no such phosphorylation could be observed in PRV-infected sensory neurons. The latter finding may explain the difference in efficiency of IFN-mediated IE suppression in HSV-1 versus PRV, since it suggests that PRV may efficiently circumvent the IFN-mediated phosphorylation of eIF2 α that normally would inhibit IE180 translation .

2. Materials and Methods

2.1. Cells and viruses

Sensory neuronal cells originating from rat dorsal root ganglion neurons (50B11) cells were a kind gift from Dr. Höke (Department of Neurology, Johns Hopkins University). The cells were grown in neurobasal medium supplemented with 1,1% glucose (20%), 0,27% L-glutamine, 10% fetal calf serum, 2% B-27 and 0,1% blasticidin (Chen et al., 2007). Differentiation of the cells was obtained by treatment with forskolin (50 μ M) (Sigma) for 24h. HSV-1 strain VR-733 (Ejercito et al., 1968) was grown and titrated on Vero cells and stored at -80°C. PRV strain Becker (Card et al., 1990) was grown and titrated on Swine Testicle (ST) cells and stored at -80°C.

2.2. Interferons and antibodies

Cells were pre-treated with a combination of 1000U/ml Universal Type I IFN- α (R&D) and
 95 100ng/ml Recombinant Rat IFN- γ (R&D) for 24h before virus inoculation. IFN remained
 present during infection. HSV-1 mouse monoclonal anti-ICP4 (sc56986) was purchased from
 Santa Cruz. The rabbit polyclonal anti-IE180 antibody was a kind gift from E. Tabarés
 (Universidad Autonoma de Madrid, Spain). Rabbit monoclonal anti-actin (A2066) was
 purchased from Sigma-Aldrich. Mouse monoclonal anti-eIF2 α (L57A5) and rabbit
 100 monoclonal anti-phospho-eIF2 α (D9G8) were purchased from Cell Signalling. HRP-
 conjugated secondary goat anti-rabbit was purchased from Cell Signaling and goat anti-mouse
 antibodies were purchased from Dako Cytomation. Thapsigargin, Hoechst 33258, and FITC-
 labeled goat anti-mouse antibodies were purchased from Invitrogen.

2.3. Total RNA isolation and quantitative RT-PCR

105 RNA from cultured cells was isolated using the Trizol Plus RNA purification kit from
 Invitrogen according to the manufacturer's protocol. Samples were treated with DNase I and
 subsequently RNA was converted into cDNA using the SuperScript III enzyme and oligodT
 primers (Invitrogen) according to the manufacturer's protocol. Subsequently quantitative PCR
 was performed using the SYBR Green dye from the SYBR Fast Bio-rad Readymix Kit
 110 (Sopachem). The primers that were used are 5' CGACACGGATCCACGACCC 3' and
 5'GATCCCCCTCCCGCGCTTCGTCCG 3' at 60°C for ICP4 (Kramer and Coen, 1995), 5'
 ACGCGAGAGGAAGTAGGGAG 3' and 5'GTACCTGCACCGCAGTGAAG 3' at 57°C for
 IE180, 5'CTGCCGTCTGGAGAAACCTG 3' and 5' CCACCACCCTGTTGCTGTAG 3' at
 53°C for the reference gene GAPDH. GAPDH expression was not affected by IFN treatment.
 115 RNA samples were prepared as four independent replicates and results of each replicate were
 normalized to GAPDH. Means and standard deviations were calculated and subsequently the

ratio of IFN-treated versus untreated values was determined. Statistical analysis was performed by using SPSS software ($p < 0,05$).

2.4. Western blotting

120 SDS/PAGE and Western blotting were performed as described previously (Deruelle et al., 2007). Blots were blocked in 5% non-fat dry milk in PBS/Tween-20 for 1h at room temperature. The blots were incubated for 1h or overnight (according to the manufacturer's instructions) with primary antibodies and washed 3 times in 0.1% TBS/Tween-20 (TBS-T). Blots were incubated with HRP-conjugated secondary antibodies for 1h at room temperature
125 and after several washing steps they were developed with enhanced chemiluminescence (ECL Plus; GE Healthcare).

2.5. Immunofluorescence staining

Cells were washed in PBS and fixed at room temperature with 3% paraformaldehyde in PBS for 10min, washed in PBS and permeabilized at room temperature in 0,2% Triton-X-100 in
130 PBS for 2min. After washing in PBS, cells were incubated for 1h at 37°C with P-eIF2 α primary antibody (1/100) diluted in PBS, washed twice in PBS and subsequently incubated for 1h at 37°C with the goat-anti-rabbit FITC secondary antibody (Invitrogen) (1/200) diluted in PBS. Afterwards cells were washed in PBS and incubated at room temperature for 10min with Hoechst 33342 (Invitrogen) (1/200) diluted in PBS. Cells were analyzed using a Nikon
135 C2 confocal microscope.

3. Results

3.1. mRNA levels of PRV IE180 and HSV-1 ICP4 are suppressed with similar and relatively late kinetics

140 Earlier, we reported that IFN-mediated suppression of ICP4 in HSV-1 and its counterpart
 IE180 in PRV correlates with establishment of *in vitro* latency in primary sensory neurons
 and that this occurs more rapid and efficient after infection with HSV-1 than with PRV (De
 Regge et al., 2010). To determine whether IFN-mediated IE suppression is noticeable at the
 mRNA level, RT-qPCR assays were performed. 50B11 sensory neuronal cells were either or
 145 not pre-treated with IFN for 24h and subsequently infected with PRV Becker or HSV-1 VR-
 733 (MOI 1). Total RNA was isolated at 4hpi, 8hpi or 12hpi and converted to cDNA followed
 by quantitative PCR. The results were normalized to GAPDH and the ratio of IE mRNA
 levels in IFN-treated versus untreated cell samples was calculated. Figure 1 shows that both
 IE180 and ICP4 mRNA levels were mildly and non-significantly reduced at 4hpi. At the later
 150 time points (8hpi and 12hpi), however, we did see a very strong and significant reduction in
 both IE180 and ICP4 mRNA expression. These data suggest that IE transcription is not
 substantially affected by IFN early in infection, allowing leaky transcription of both ICP4 and
 IE180 early in infection. Later in infection (>4hpi), a substantial suppression of IE mRNA,
 suggestive for a block in transcription, was observed for both HSV-1 and PRV. No apparent
 155 differences could be observed between HSV-1 and PRV.

3.2. Protein levels of PRV IE180 are suppressed later compared to HSV-1 ICP4

To determine whether the relatively slow IFN-mediated suppression of ICP4 and IE180
 mRNA levels correlate with the kinetics of IE protein levels in sensory neuronal cells,
 Western blot assays were performed. 50B11 cells were either or not pre-treated with IFN for
 160 24h and subsequently infected with PRV Becker or HSV-1 VR-733 (MOI 1). Cell lysates
 were collected at 4h, 8h and 12hpi and immunoblotting was performed for IE180 and actin
 (loading control) for PRV samples and ICP4 and actin (loading control) for HSV-1 samples.
 For HSV-1, IFN treatment resulted in a rapid and strong reduction of ICP4 protein expression
 at 4hpi (Fig. 2). For PRV, however, IFN-mediated IE180 protein suppression was markedly

165 slower. IE180 protein levels were not noticeably suppressed at 4hpi and were only substantially reduced later in infection (8hpi and 12hpi)(Fig. 2). These results implicate a rapid translational inhibition of HSV-1 ICP4 compared to a markedly slower suppression of PRV IE180 protein levels.

3.3. PRV strongly suppresses IFN-induced phosphorylation of eIF2 α

170 Our results suggest that IFN-mediated translational shutdown in 50B11 cells is markedly more rapid and efficient for HSV-1 ICP4 compared to PRV IE180. Through activation of protein kinase R (PKR), IFN induces phosphorylation of the translation initiation factor eIF2 α , which shuts down translation. To determine whether the observed differences in IFN-mediated HSV-1 ICP4 versus PRV IE180 protein suppression in 50B11 cells correlate with
175 differences in IFN-mediated phosphorylation of the translation initiation factor eIF2 α , immunoblotting was performed. Thapsigargin, an inhibitor of sarco/ER Ca²⁺ ATPases that induces phosphorylation of eIF2 α , was included as a positive control for phosphorylation of eIF2 α . 50B11 cells were either or not pre-treated with IFN for 24h and subsequently infected with PRV-Becker or HSV-1 VR-733 (MOI 1). Lysates were collected at 4hpi and Western
180 blotting was performed for eIF2 α and phosphorylated eIF2 α (P-eIF2 α), ICP4 (in HSV-1 infected samples) and IE180 (in PRV infected samples). Figure 3.A. shows a substantial phosphorylation of eIF2 α in IFN-treated samples infected with HSV-1, but not in PRV-infected cells, corresponding with the clear reduction of ICP4 protein expression and the lack of IE180 protein reduction in IFN-treated samples. To confirm these results, an
185 immunofluorescence staining was performed on cells that were either untreated, treated with 1 μ M thapsigargin for 1h, or treated with 1 μ M thapsigargin for 1h and subsequently infected with PRV or HSV-1 (MOI 1). At 4hpi cells were fixed and stained for phosphorylated eIF2 α . Figure 3.B. shows that the thapsigargin treatment effectively stimulates the phosphorylation of eIF2 α . PRV infection of thapsigargin-treated cells resulted in a strong reduction of

phosphorylated eIF2 α . Infection of thapsigargin-treated cells with HSV-1 also reduced the level of eIF2 α phosphorylation compared to the thapsigargin treated mock cells, but to a much lesser extent. Hence, in 50B11 cells, PRV efficiently counteracts both the IFN-induced and thapsigargin-induced phosphorylation of the translation initiation factor eIF2 α .

4. Discussion

The immediate early (IE) protein ICP4 in HSV-1 and its ortholog IE180 in PRV are the first viral proteins produced during infection of a host cell and are of central importance in the onset of a lytic alphaherpesvirus replication as they act as transactivators of the viral genome (DeLuca and Schaffer, 1985). Hence, to enable the establishment of latency in sensory neurons, ICP4/IE180 expression needs to be shut down. Recently, we reported that IFN is able to suppress ICP4 and IE180 protein levels in sensory neurons to undetectable levels, which correlated with the establishment of virus latency *in vitro* (De Regge et al., 2010). IFN-mediated suppression was less efficient for IE180 compared to ICP4, which correlated with less efficient establishment of *in vitro* latency for PRV versus HSV-1 (De Regge et al., 2010).

Here, we provide mechanistic insights in the IFN-mediated suppression of ICP4 and IE180 in sensory neuronal cells. We observed that levels of ICP4/IE180 mRNA were mildly but not significantly reduced by IFN at 4hpi, although we did observe a strong ICP4 protein shutdown at that time point. This indicates that IE transcription is not immediately suppressed by IFN in sensory neuronal cells, allowing some ICP4/IE180 mRNA to be produced. At later stages of infection (8hpi and 12hpi), however, we did observe a substantial reduction in ICP4/IE180 mRNA levels in IFN-treated samples, suggesting a transcriptional block at these time points. A transcriptional block of HSV-1 and PRV immediate early expression has been reported before, although the underlying mechanism remains unknown (Gloger and Panet, 1984; Oberman and Panet, 1988; De Stasio and Taylor, 1990; Nicholl and Preston, 1996; Yao

et al., 2007). Several potential explanations could be given to explain such a transcriptional
 215 block. On the one hand, IFN may affect expression levels of cellular proteins involved in viral
 DNA transcription. Initial transcription of alphaherpesviruses is regulated by a transcription
 complex that comprises the viral VP16 protein and the cellular Oct-1 and HCF1 (Hagmann et
 al., 1995). It has been reported that IFN α causes a downregulation of Oct-1 and Oct-2
 resulting in a repressed transcription (Dent et al., 1991). Other IFN-mediated effects on
 220 transcription have mostly focussed on RNA viruses and include IFN-induced proteins like
 APOBEC3G, TRIM5 α , and ISG20 (Liu et al., 2010). Whether these or other IFN-induced
 effectors may also interfere with transcription of DNA viruses will be interesting to further
 investigate. On the other hand, IFN has been reported to induce activity of RNase L, which
 may lead to IE mRNA degradation (Liu et al., 2010). Arguing against this possible
 225 explanation is our observation that expression of the reference gene GAPDH was not reduced
 in IFN-treated samples (data not shown). This either suggests that the effects of IFN on
 ICP4/IE180 mRNA cannot be (solely) attributed to IFN-induced RNA degradation or that
 such degradation is selective towards viral transcripts.

For HSV-1, we observed a fast and efficient IFN-induced suppression in ICP4 protein levels
 230 in sensory neuronal cells (4hpi), before substantial shutdown of mRNA levels (from 8hpi
 onwards). This may indicate that in HSV-1, ICP4 transcripts that are present at 4hpi are not
 efficiently translated into protein. One of the most potent effects of IFN on translation is
 phosphorylation and thereby inactivation of the translation initiation factor eIF2 α through
 protein kinase R (PKR) (Liu et al., 2010). Accordingly, in HSV-1-infected sensory neuronal
 235 cells, we observed a marked, IFN-induced phosphorylation of eIF2 α . For PRV, on the other
 hand, we did not observe eIF2 α phosphorylation. Probably as a result of this, PRV IE180
 protein levels were not reduced by IFN at 4hpi. The apparent ability of PRV to counteract
 phosphorylation of eIF2 α and the concomitant inability of IFN to efficiently shut down IE180

protein levels correlate well with our earlier findings that IFN results in less efficient suppression of PRV IE180 compared to HSV-1 ICP4 and concomitant less efficient establishment of PRV versus HSV-1 latency in *in vitro* cultures of sensory neurons (De Regge et al., 2010). Nevertheless, our finding that HSV-1 is less capable to suppress eIF2 α phosphorylation than PRV in sensory neuronal cells was somewhat surprising. Indeed, HSV-1 has been reported to encode a potent factor that interferes with eIF2 α phosphorylation, ICP34.5, which functions as an antagonist of the PKR response by redirecting the host protein phosphatase 1 α to dephosphorylate eIF2 α (He et al., 1997). To exclude the possibility that ICP34.5 expression was lost by cell culture adaptation of the HSV-1 strain used or other reasons, Western blots showed abundant ICP34.5 expression upon infection of 50B11 cells with HSV-1 (data not shown). In line with this are our findings that HSV-1 is able to suppress phosphorylation of eIF2 α to some extent in 50B11 cells treated with thapsigargin, albeit markedly less efficient than PRV (Figure 3.B.). Despite the fact that, under the current experimental conditions, PRV prevented IFN-induced eIF2 α phosphorylation more efficiently than HSV-1, PRV does not encode an ICP34.5 ortholog. Future research will aim at identifying the viral factor(s) involved.

In conclusion, IFN is able to suppress HSV-1 ICP4 and PRV IE180 levels in sensory neuronal cells by i) reducing the transcription of these viral genes, relatively late in infection and ii) for HSV-1, preventing translation of ICP4 early in infection likely through the phosphorylation of eIF2 α . In addition, PRV is able to efficiently inhibit phosphorylation of eIF2 α through an unknown mechanism.

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7. Figure Captions

Fig.1: IFN suppresses mRNA levels of PRV IE180 and HSV-1 ICP4 with similar kinetics. 50B11 cells were pre-treated with IFN for 24h before virus inoculation. Total RNA samples were isolated at 4hpi, 8hpi or 12hpi with (A) PRV Becker or (B) HSV-1 VR-733 (MOI 1). RNA was converted to cDNA followed by RT-qPCR for GADPH, IE180 (PRV)

and ICP4 (HSV-1). Viral mRNAs were normalized to GAPDH and the fold change was determined by calculating the ratio between IFN-treated and untreated samples.

Fig.2 : Protein levels of PRV IE180 are suppressed later compared to HSV-1 ICP4 upon

340 **IFN treatment.** 50B11 cells were either or not pre-treated with IFN for 24h before virus inoculation. Cell lysates were collected at 4hpi, 8hpi or 12hpi with (A) PRV Becker or (B) HSV-1 VR-733 (MOI 1). Western blotting was performed for actin, IE180 (PRV) and ICP4 (HSV-1).

Fig.3: PRV strongly suppresses IFN-induced phosphorylation of eIF2 α . (A) 50B11 cells

345 were either or not pre-treated with IFN for 24h before virus inoculation. Cell lysates were collected at 4hpi with PRV Becker or HSV-1 VR-733 (MOI 1). Western blotting was performed for total eIF2 α , P-eIF2 α , IE180 (PRV) and ICP4 (HSV-1). Thapsigargin treatment (1 μ M for 2h) was included as a positive control to induce phosphorylation of eIF2 α . (B) 50B11 cells were either or not pre-treated with thapsigargin (1 μ M for 1h) before inoculation
350 with PRV Becker or HSV-1 VR-733 (MOI 1). Cells were fixed at 4hpi and immunofluorescence staining was performed for P-eIF2 α (green). Nuclei were counterstained with Hoechst 33258 (blue).

Figure 1

Figure 2

Figure 3