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Interferon-induced inhibition of parainfluenza virus type 5; the roles of MxA, PKR and oligo A synthetase/RNase L

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Abstract

We have previously reported that the addition of interferon (IFN) to the culture medium of Vero cells (which cannot produce IFN) that were infected with the CPI− strain of parainfluenza virus 5 (PIV5, formally known as SV5), that fails to block IFN signaling, rapidly induces alterations in the relative levels of virus mRNA and protein synthesis. In addition, IFN treatment also caused a rapid redistribution of virus proteins and enhanced the formation of cytoplasmic viral inclusion bodies. The most studied IFN-induced genes with known anti-viral activity are MxA, PKR and the Oligo A synthetase/RNase L system. We therefore examined the effects of these proteins on the replication cycle of PIV5. These studies revealed that while these proteins had some anti-viral activity against PIV5 they were not primarily responsible for the very rapid alteration in virus protein synthesis observed following IFN treatment, nor for the IFN-induced formation of virus inclusion bodies, in CPI− infected cells.

Keywords: Parainflueza virus type 5; Interferon; PKR; OAS; MxA

Introduction

Parainfluenza virus type 5 (previously known as simian virus 5; SV5) (Chatziandreou et al., 2004), is a prototype member of the *Rubulavirus* genus in the *Paramyxovirinae* subfamily of the family *Paramyxoviridae* (Lamb and Kolakofsky, 2001). PIV5, like all other paramyxoviruses, is an enveloped, non-segmented negative-stranded RNA virus. The helical nucleocapsid (rather than free genomic RNA) acts as a template for all RNA synthesis. The viral polymerase complex transcribes, in a sequential manner, the NP, V/P, M, F, SH, HN and L genes, after polymerase entry at the single 3′ promoter of the template, by a "stop–start" transcription mechanism. Efficiency of transcription decreases with increasing distance of the genes from the promoter generating a transcriptional gradient, with the NP gene transcribed most frequently and the L gene transcribed the least frequently (reviewed in Whelan et al., 2004).

Interferon (IFN)-α/β are produced by cells in direct response to virus infection and are characterized by their ability to induce antiviral responses and cell growth inhibitory effects. The secreted IFN-α/β bind to the IFN-α/β receptor on the surface of the infected cells and neighboring cells to initiate an intracellular signaling cascade that ultimately activates the expression of hundreds of IFN-inducible genes. Some of the genes up-regulated by IFN stimulation are involved in the establishment of an antiviral state. Well characterized examples of IFN-induced antiviral proteins include protein kinase R (PKR), the MxA GTPases and the family of 2′-5′ oligoadenylate synthetases (OAS) that activate the latent endoribonuclease L (RNase L). PKR is a dsRNA activated, serine–threonine protein kinase normally present in the cell in an inactive form (Williams, 1999). PKR can mediate inhibition of protein synthesis through phosphorylation of eukaryotic initiation factor-2α (eIF-2α), which, in the course of viral infection, provides a defence mechanism for restricting viral protein translation and, ultimately, viral replication (Clemens and Elia, 1997; Meurs et al., 1990). Mx proteins are dynamin-like large GTPases that have antiviral activity and inhibit the multiplication of several RNA viruses, in contrast to other IFN-stimulated genes, they are not constitutively expressed in cells. The importance of Mx proteins for host survival has been amply demonstrated (Arnheiter et al.,

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1996; Hefti et al., 1999; Kochs et al., 2002; Pavlovic et al., 1995), but the mechanism of MxA antiviral action is still not completely understood. The viral target recognised by MxA is virus- and cell type-specific, and it can inhibit virus transcription or mRNA translation, or interfere with viral ribonucleocapsid protein complexes or transportation of virus nucleocapsids (Haller and Kochs, 2002). The OAS system consists of enzymes that, when activated by dsRNA, catalyze the synthesis of oligoadenylates whose function is to activate latent RNase L, which degrades ssRNA, including both viral and cellular mRNA, thereby blocking protein synthesis and leading to viral inhibition (Zhou et al., 1997).

Many paramyxoviruses have been shown to at least partially circumvent the IFN response by blocking IFN signaling and limiting IFN production. PIV5 blocks IFN signaling by targeting STAT1, a host cell transcription factor essential for both IFN-α/β and IFN-γ signaling, for proteasome-mediated degradation (Didcock et al., 1999a, 1999b). The V protein of PIV5 also helps to limit IFN production by interacting with, and inhibiting the action of, mda-5, an intracellular signaling molecule which plays a key role in at least one intracellular signaling pathway that leads to the induction of IFN (Andrejeva et al., 2004). However, the ability of PIV5 to circumvent the IFN response is not absolute as infected cells still release some IFN, which can induce an antiviral state in neighboring uninfected cells, thereby restricting the replication of PIV5 (Andrejeva et al., 2002; Chatziandreou et al., 2004; Didcock et al., 1999a; Wansley et al., 2005). In support of these observations, PIV5 replication was shown to be enhanced in cells that have been engineered to be non-responsive to IFN (Young et al., 2003). We have recently described a model in which it is possible to study the kinetics of IFN-induced effects on PIV5 transcription, protein synthesis and the distribution of virus proteins, in the absence of virus countermeasures. In this model, CPI−, a canine strain of PIV5 that fails to block IFN signaling, was used to infect Vero cells, which are unable to produce IFN due to spontaneous gene deletions (Desmyter et al., 1968; Mosca and Pitha, 1986) but can respond to exogenous IFN supplemented to culture medium. It was shown that addition of IFN to CPI− infected cells, once virus replication was established, rapidly changed the profile of virus transcription and protein synthesis. IFN increased the steepness of the virus mRNA transcription gradient and the production of virus mRNAs with longer poly(A) tails, which suggests that the virus polymerase processivity may be altered in cells in an IFN-induced antiviral state (Carlos et al., 2005). Although not in complete concordance with mRNA levels, IFN also caused an alteration in the protein synthesis pattern such that there was a marked down-regulation in the expression levels of genes downstream of the V/P gene. Furthermore, IFN treatment led to a redistribution of virus proteins within infected cells that resulted in the formation of inclusion bodies (Carlos et al., 2005). In this report we attempt to further define how IFN mediates these effects, and demonstrate that although PKR, oligo A/RNase L and MxA have some anti-viral activity none of them appears to be primarily responsible for the rapid IFN-induced alterations in the PIV5 replication cycle.

Results

Comparison of the effects of IFN on CPI− virus protein synthesis in Hep2 and Vero cells

To ascertain whether similar IFN-induced changes in the pattern of protein synthesis observed in Vero cells infected with CPI− occurred in cells that can produce and respond to IFN, we examined the replication of CPI− in human Hep2 cells. Vero and Hep2 cells were mock infected or infected with CPI− or CPI+ (the parental virus that blocks IFN signalling) and either treated with IFN at 12 h p.i. or left untreated. Six hours after IFN treatment, cells were metabolically labeled with [^{35}S] methionine for 1 h and the relative levels of newly synthesized viral proteins were estimated by immunoprecipitation (Figs. 1A and B). It was

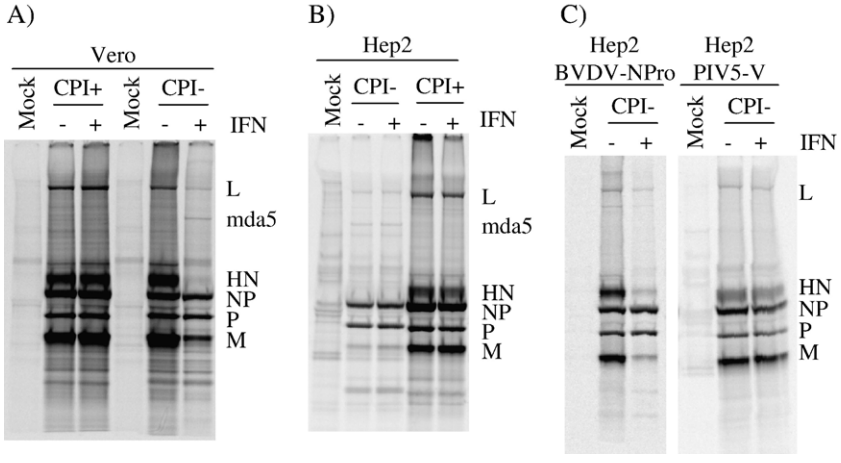

Fig. 1. CPI− protein synthesis profile in Vero, Hep2 naive, Hep2/BVDV-NPro and Hep2/PIV5-V cells, in the absence and presence of IFN. Vero (A) and Hep2 naïve cells (B) were infected with CPI− or CPI+, and Hep2/BVDV-NPro and Hep2/PIV5-V cells (C) were infected with CPI−. Cells were infected at m.o.i. of 50 pfu/cell and either treated with exogenous rHuIFN-α at 12 h p.i. or left untreated. Cells were metabolically labeled with [^{35}S]-methionine for 1 h, 6 h after addition of IFN. Virus proteins were immunoprecipitated from extracts of these cells with a pool of antibodies to the NP, P, M, HN and L proteins. The precipitated proteins were subsequently separated on a 4–12% gradient PAG and visualized by phosphorimager analysis.

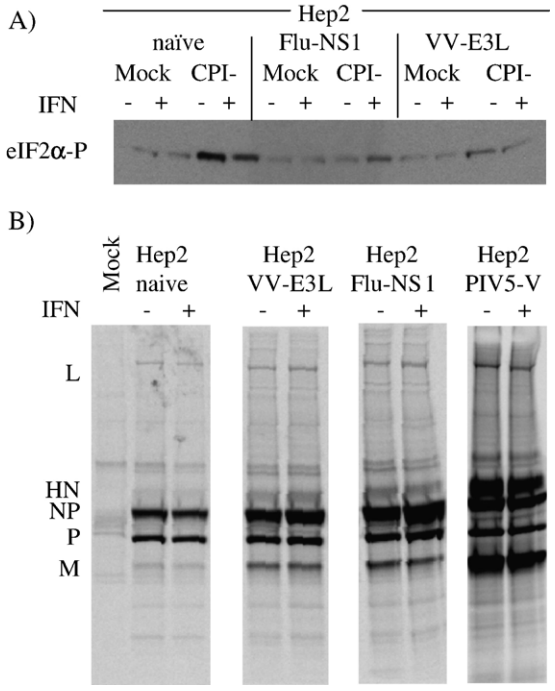

Fig. 2. Effect of VV E3L or influenza virus NS1 expression on CPI− protein synthesis in Hep2 cells. Hep2 naïve, Hep2/E3L, Hep2/NS1 and Hep2/PIV5-V cells were infected with CPI− at an m.o.i. of 50 pfu/cell and 12 h later treated with IFN (or left untreated). Detection of phosphorylated eIF-2α (eIF-2α-P) by immunoblot analysis (A). Metabolic labeling with [^{35}S]-methionine for 1 h at 18 h p.i. and immunoprecipitation of viral proteins (B). The labeled proteins were separated by SDS-PAGE and visualized by phosphorimager analysis.

clear from these results that the pattern of CPI− virus protein synthesis in untreated Hep2 cells was very similar to that observed in Vero cells infected with CPI− and treated with IFN. Thus, while the level of expression of NP, P and V was high, there was markedly reduced M, HN and L expression. Given that Hep2 cells produce IFN upon virus infection, it was not surprising that addition of exogenous IFN at 12 h p.i. cells had no additional effect on the pattern of viral protein synthesis. In contrast, and as expected given that CPI+ blocks IFN signaling, the pattern of CPI+ protein synthesis was not affected in Hep2 cells, even though these cells can produce and respond to IFN. To confirm that the unusual pattern of CPI− protein synthesis was due to Hep2 cells producing and responding to IFN we engineered Hep2 cells to constitutively express either a functional V protein of PIV5 that blocks IFN signaling (Hep2/PIV5-V cells) or NPro of BVDV (Hep2/BVDV-NPro), which targets IRF-3 for proteasome degradation and thus blocks IFN production (Hilton et al., 2006). In contrast to naive Hep2 cells, a normal pattern of CPI− virus protein synthesis was observed in Hep2/PIV5-V cells, both in the presence and absence of exogenous IFN, as well as in Hep2/BVDV-NPro cells in the absence of IFN. However, in a similar manner to that observed in Vero cells, addition of exogenous IFN to Hep2/BVDV-NPro cells induced a rapid change in the pattern of CPI− virus protein synthesis (Fig. 1C).

The role of PKR and oligo A synthetase/RNase L in altering the pattern of CPI− protein synthesis

As part of the ongoing studies within our group, Hep2 polyclonal cell lines expressing IFN antagonists have been isolated, these include the E3L (Hep2/E3L; Hilton et al., 2006) and NS1 (Hep2/NS1) proteins of vaccinia virus and Influenza A virus (strain PR8; Hale et al., 2006), respectively. The E3L and NS1 proteins both bind dsRNA and interact with PKR to inhibit its activity. To determine whether constitutive expression of either NS1 or E3L in Hep2 cells inhibited the activity of PKR, we examined whether or not eIF2-α was phosphorylated in these cells following infection with CPI−. These results clearly showed that the levels of phosphorylated eIF-2α were strongly reduced in Hep2/E3L and Hep2/NS1 cells compared to Hep2 naive cells (Fig. 2A). Therefore we next compared the patterns of CPI− protein synthesis in Hep2/E3L and Hep2/NS1 cells to naive Hep2 cells and Hep2 cells constitutively expressing the V protein of PIV5 (Hep2/PIV5-V). The cells were infected with CPI− at a high m.o.i., treated with IFN at 12 h p.i. (or left untreated) and 6 h later the cells were metabolically labeled with [^{35}S]-methionine for 1 h. The relative levels of virus protein synthesis were estimated by immunoprecipitation (Fig. 2B). Although the ratio of M and HN proteins compared to NP and P proteins synthesized in Hep2/E3L and Hep2/NS1 cells was slightly higher than in naive Hep2 cells, the expression of M and HN was significantly reduced compared to Hep2/PIV5-V cells. These results suggest that although inhibition of PKR may weakly enhance viral gene expression it does not appear to be primarily responsible for the IFN-induced changes in CPI− virus protein synthesis.

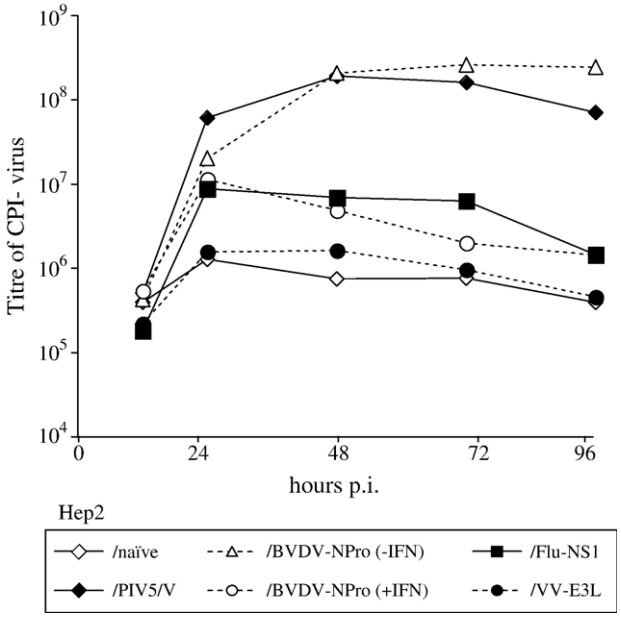

Fig. 3. CPI− virus yield in naïve Hep2, Hep2/PIV5-V, Hep2/E3L, Hep2/NS1, Hep2/BVDV-NPro cells. Amount of infectious virus released from naïve Hep2 cells and from various Hep2 cells expressing different IFN antagonists, which have been infected with CPI− at high m.o.i. Hep2/BVDV-NPro cells were or were not treated with exogenous IFN at 12 h p.i.

Given that IFN clearly alters the general pattern of CPI− protein synthesis in Hep2 cells, we next compared the production of infectious virus in naive Hep2 cells and in Hep2 cells constitutively expressing the different IFN antagonists (Fig. 3). Cells were infected at high m.o.i. and the amount of virus released monitored up to 72 h p.i. These results showed that there was approximately 100-fold increase in the titer of CPI− in Hep2 cells that had been engineered either not to produce IFN (Hep2/BVDV-NPro cells) or respond to IFN (Hep2/PIV5-V cells) compared to either naive Hep2 cells or Hep2/BVDV-NPro cells treated with exogenous IFN at 12 h p.i. Expression of the NS1 protein of influenza virus improved the yield approximately 10-fold. However, expression of E3L did not significantly improve virus growth.

These results suggested that PKR was not responsible for the major alterations in virus protein synthesis observed when CPI− infected cells were exposed to IFN. Furthermore, since both E3L and NS1 have also been reported to inhibit the oligo A/RNase L system (Beattie et al., 1995; Min and Krug, 2006), these results also imply that the oligo A/RNase L system is not responsible for these IFN-induced effects on the replication cycle of PIV5. In order to address this question further, we examined the affect of IFN on the pattern of CPI− virus protein synthesis in naive Hep2 and Hep2/BVDV-NPro cells that had or had not been treated with siRNA to PKR or RNase L prior to infection (Fig. 4). siRNA treatment successfully reduced the levels of both PKR and RNase L and also prevented the levels of PKR and RNase L increasing upon IFN treatment. Nevertheless, treatment of cells with siRNA to PKR or RNase L did not prevent IFN treatment from rapidly altering the pattern of virus protein synthesis, in a similar manner to that observed in siRNA-untreated Hep2/BVDV NPro cells. Furthermore, the M, HN or L levels did not increase in siRNA-treated naive cells compared to untreated cells (Fig. 4). These results also suggest that the IFN-induced rapid change in the pattern of CPI− protein synthesis is not due to the antiviral actions of PKR or RNase L.

MxA reduces the yield of virus but is not responsible for the rapid alteration in CPI− virus protein synthesis induced by IFN

Since Hep2 cells are deficient in MxA expression (Fig. 5A) the results presented above also suggested that MxA may not play a significant role in the gross changes seen in the pattern of CPI− in the presence of IFN. However, IFN not only induces a change in CPI− virus protein synthesis but also induces a redistribution of virus nucleocapsid proteins into large cytoplasmic inclusion bodies. Since it has been reported for other viruses, including La Crosse virus, that MxA sequesters nucleocapsids into elongated tubular structures resulting in cytoplasmic inclusions, we examined in more detail how MxA affects the CPI− replication cycle. In these experiments the replication of CPI− in naive Vero cells, that do not express MxA in the absence of IFN, was compared with Vero cells that have

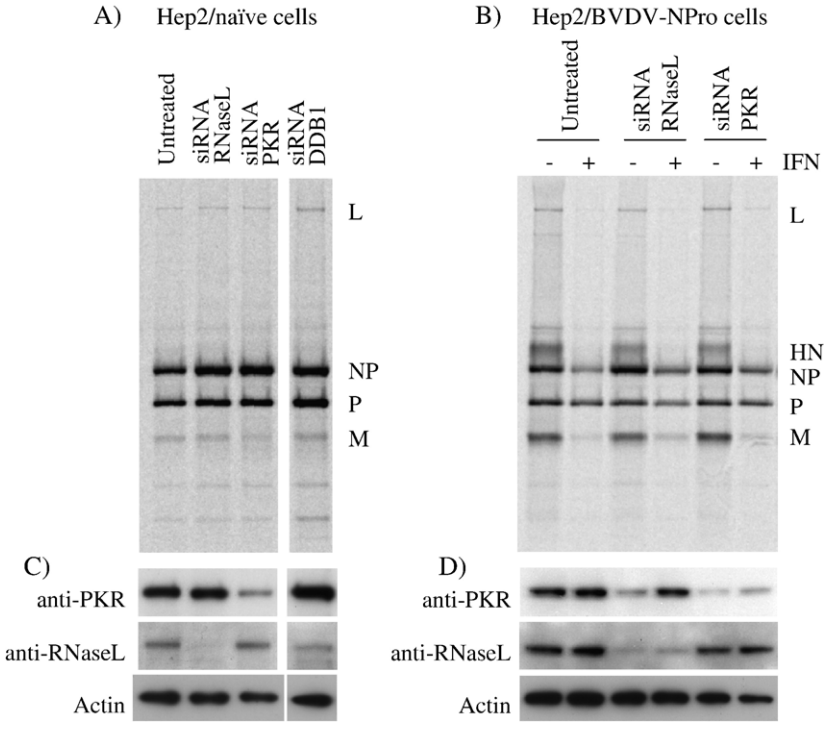

Fig. 4. Effect of siRNA knockdown of RNase L or PKR on CPI− protein synthesis in naïve Hep2 and Hep2/BVDV/NPro cells. (A and B) Naïve Hep2 and Hep2/BVDV-NPro cells, respectively, were treated with either siRNAs to RNase L, PKR or DDB1 (control) for 30 h. The cells were then infected with CPI− at high m.o.i. (50 pfu/cell) and exogenous IFN was or was not supplemented to Hep2/BVDV-NPro cells at 12 h p.i. The cells were metabolically labeled with [^{35}S]-methionine for 1 h at 18 h p.i.. The labeled viral proteins were immunoprecipitated, separated by SDS-PAGE and visualized by phosphorimager analysis. (C and D) The depletion of RNase L or PKR and levels of actin were monitored by immunoblot analysis of total cell extracts (of the same infected cell extracts used in panels A and B, respectively) using specific antibodies for these proteins.

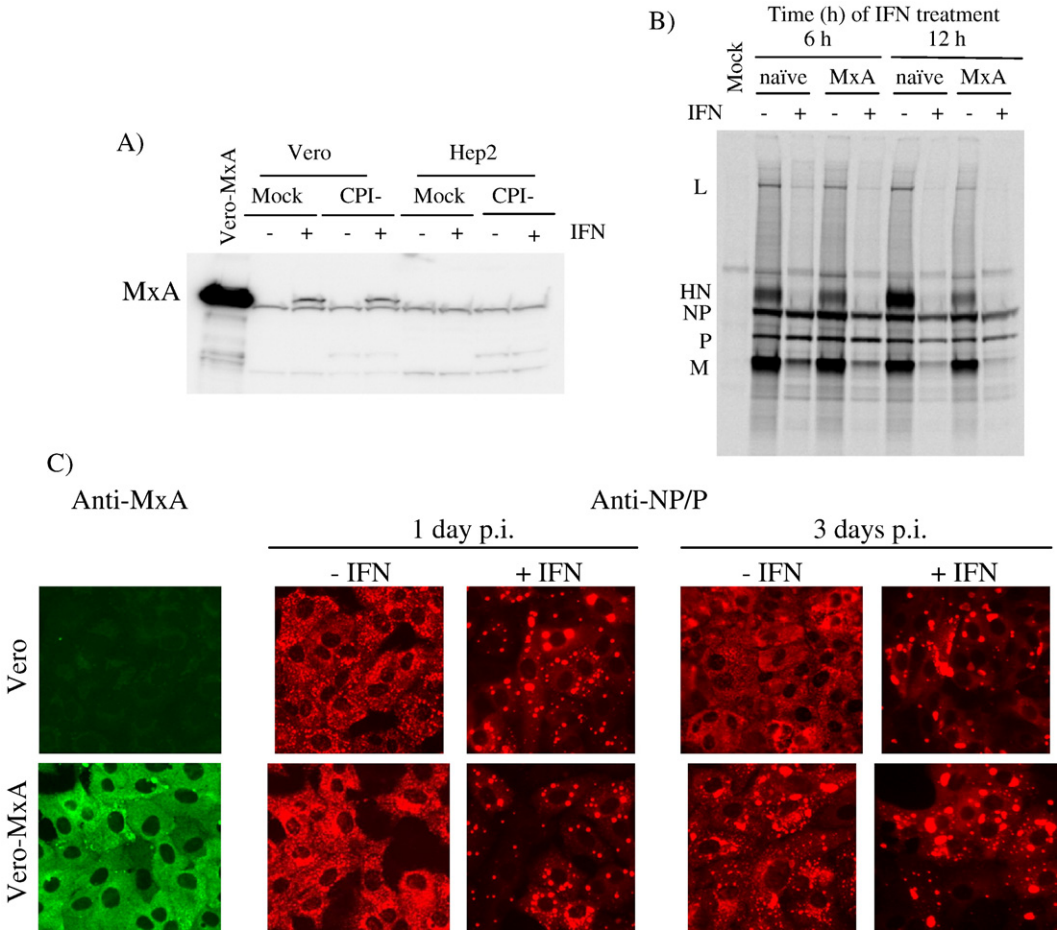

Fig. 5. MxA is not responsible for the observed decrease of the relative levels of CPI− protein synthesis in the presence of IFN. (A) Hep2 cells do not express endogenous MxA protein. Vero cells and Hep2 cells were mock-infected or infected with CPI− at high m.o.i. (50 pfu/cell) and treated with IFN at 8 h p.i. or left untreated. At 24 h p.i. the cells were lysed and the proteins contained in total cell extracts were separated by SDS-PAGE. Immunoblot analysis was subsequently performed using polyclonal anti-MxA antibody. (B) Naïve cells and MxA-expressing Vero cells were infected with CPI− at an m.o.i. of 50 pfu/cell, and at 12 h p.i. the cells were, or were not, treated with exogenous IFN. Six and twelve hours later cells were metabolically labeled with [^{35}S]-methionine for 1 h. Virus proteins were immunoprecipitated, separated by SDS-PAGE and visualized by phosphorimager analysis. (C) Naïve and MxA-expressing Vero cells were infected with CPI− at high m.o.i. (50 pfu/cell) and at 12 h p.i. the medium was (+IFN), or was not (−IFN), supplemented with IFN. The cell monolayers were fixed at 1 and 3 days p.i. and stained for MxA and NP/P.

been engineered to constitutively express high levels of MxA (Frese et al., 1996; Stertz et al., 2006; Fig. 5B). Naive and MxA-expressing Vero cells were infected with CPI− at a high m.o.i. and 12 h later IFN was, or was not, added to the culture medium. At 6 and 12 h after IFN treatment, the cells were metabolically labeled with [^{35}S]-methionine and the relative levels of virus proteins were estimated by immunoprecipitation (Fig. 5B). Although in the absence of IFN treatment there was a slight apparent reduction in HN expression in MxA-expressing cells, the pattern of virus protein synthesis remained relatively normal in the MxA-expressing Vero cells. However, in both cell types IFN induced a rapid change in the pattern of virus protein synthesis such that there was an obvious reduction in the relative levels of M, HN and L protein synthesis, but not NP and P.

Although these studies showed that MxA does not have a dramatic effect on CPI− protein synthesis, it may still affect other stages of the PIV5 replication cycle, such as the distribution of NP and P proteins. To address this question, naive Vero and MxA-expressing Vero cells were infected with CPI− and were, or were not, treated with IFN at 12 h p.i. At 1 and 3 days p.i. the cells were fixed and stained for the cellular MxA protein and the viral NP and P proteins. As shown in Fig. 5C, MxA does not induce inclusion body formation at 1 day p.i. and the relatively diffuse distribution of NP and P proteins observed in MxA-expressing Vero cells was similar to that observed in naive infected cells. However, following the addition of IFN to both naive and MxA-expressing cells, NP and P proteins redistributed into distinct cytoplasmic inclusion bodies. Furthermore, MxA did not obviously co-localize with the viral inclusion bodies and was diffusely distributed throughout the cytoplasm both in IFN-treated and untreated cells (data not shown). However, by 3 days p.i., there was a clear difference in the distribution of NP and P proteins in MxA-expressing cells in comparison with non-MxA-expressing cells; there was more NP and P in inclusion bodies when MxA was being expressed (Fig. 5C, right panel), although there was still no apparent co-localization of MxA with the NP and P protein accumulations (data not shown).

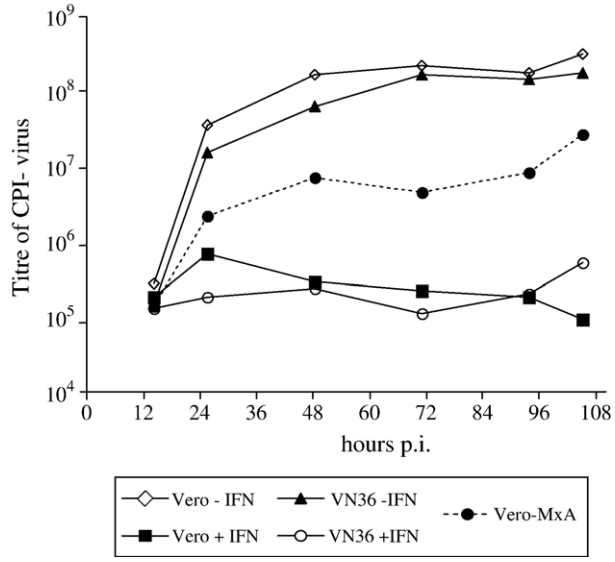

Fig. 6. Effect of MxA and IFN on CPI− virus yield. Amount of infectious virus released from naive and Vero-control (VN36) cells that were or were not treated with IFN at 12 h p.i., and from MxA-expressing Vero cells, that had been infected with CPI− at high m.o.i. (50 pfu/cell).

Although we could see no obvious effects of MxA at early times post infection, immunofluorescence data suggested that MxA may be affecting virus replication at later times. To ascertain whether this late effect may influence the production of infectious virus, naive and MxA-expressing Vero cells, as well as an additional control Vero cell-line termed VN36 (that was generated at the same time as the MxA-expressing cells using a non-expressing plasmid; Frese et al., 1995) were infected with CPI− at a high m.o.i. and virus growth was determined (Fig. 6). The results obtained showed that, in the absence of exogenous IFN, CPI− virus titers were reduced approximately 10-fold in MxA-expressing cells in comparison to naive cells (Fig. 6). However, addition of IFN at 12 h p.i. had a stronger effect on CPI− growth, such that by 108 hrs p.i. CPI− virus titers in IFN treated cells were reduced approximately 1000-fold in comparison to untreated cells (Fig. 6).

Discussion

We have previously reported that IFN alters the replication cycle of PIV5 in Vero cells by inducing an antiviral response which changes the pattern of PIV5 transcription and protein synthesis and results in an altered distribution of virus proteins (Carlos et al., 2005). To determine whether the same IFN-induced anti-viral mechanisms operate in other cells, we engineered Hep2 cells to constitutively express BVDV NPro, which targets IRF-3 for proteasome-mediated degradation and thus blocks the ability of the cells to produce IFN in response to virus infection (Hilton et al., 2006). However, since NPro does not block IFN signaling, the engineered cells can still respond to exogenous IFN. In Hep2/BVDV-NPro cells infected with CPI− a normal pattern of virus protein synthesis was observed, but this was rapidly altered, in a manner similar to that observed in Vero cells infected with CPI−, upon the addition of IFN to the culture media. In naive Hep2 cells, because they produce and respond to IFN in response to CPI− infection, this IFN-induced pattern of virus protein synthesis was seen even without addition of exogenous IFN. Furthermore, we have observed similarly altered patterns of CPI− protein synthesis in a variety of tissue culture cells that produce and respond to IFN (data not shown). The question therefore remains as to whether this IFN-induced alteration in the pattern of virus protein synthesis is simply an unavoidable consequence of the mode of replication of PIV5 (and other rubulaviruses/paramyxoviruses; Carlos et al., 2005), or whether it has been evolutionarily selected for, perhaps either to ensure sufficient V protein is made to dismantle the IFN-induced anti-viral state of cells (by rapidly targeting STAT1 for degradation (Didcock et al., 1999b; unpublished observations), or to allow PIV5 to establish prolonged or persistent infections as we have previously suggested (Carlos et al., 2005).

IFN-α/β activate the expression of more than 300 ISGs, many of which have direct or indirect anti-viral activities. Of those with proven antiviral activity, PKR, OAS and Mx proteins are probably the best studied. Indeed, transgenic mice deficient in these responses are in general more susceptible to a variety of viruses (Abraham et al., 1999; Arnheiter et al., 1996; Zhou et al., 1997). However, mice lacking PKR, RNase L and Mx are still capable of mounting a limited IFN-induced anti-viral response, demonstrating that there must be additional IFN-induced anti-viral proteins (Zhou et al., 1999). The results presented here are thus somewhat surprising in that they demonstrate that PKR, OAS/RNase L and MxA alone are not responsible for the major changes in PIV5 transcription and protein synthesis observed in cells in an IFN-induced anti-viral state, although we cannot rule out the possibility that they may act in concert with other IFN-induced proteins to mediate these effects. Nevertheless, MxA appears to have some anti-PIV5 activity as, at late times p.i., there was a difference in the distribution of the NP and P proteins in Vero and Vero-MxA cells, suggesting that MxA can directly or indirectly induce late formation of inclusion bodies. Furthermore, the yield of virus from MxA-expressing Vero cells was ten-fold lower than from naive Vero cells. The antiviral activities of PKR and RNase L against PIV5 are less obvious. Thus, although the expression levels of M, HN, and L proteins were enhanced in Hep2/VV-E3L and Hep2/Flu-NS1 cells (in which PKR activity was inhibited), their levels were still significantly lower than in Hep2/BVDV-NPro or Hep2/PIV5-V cells. Furthermore, treatment of Hep2 cells, or Hep2/BVDV-NPro cells with siRNA to RNase L or PKR prior to infection did not alter the IFN-induced pattern of CPI− protein synthesis. On the other hand the yield of virus from Hep2/Flu-NS1 cells was a ten-fold higher than in naive Hep2 cells. However, since NS1 is a multifunctional protein, it is possible that the improvement in CPI− replication observed in Hep2/Flu-NS1 cells may be due to another property of NS1, e.g. its ability to activate PI3 kinase (Hale et al., 2006) or limit IFN production (Garcia-Sastre et al., 1998), rather than its ability to block PKR (or oligo A/RNase L) activity. Thus it is still not clear which IFN-induced proteins are responsible for the changes in PIV5 transcription and protein synthesis. However, since IFN primarily induces a specific alteration in the pattern of PIV5 protein synthesis (rather than

simple proportionate reduction in the levels of synthesis of all virus proteins), it is likely that this IFN-induced effect will be mediated by a protein(s) which specifically alter(s) a virus component of the PIV5 replication cycle, e.g. the polymerase (Carlos et al., 2005), rather than proteins, such as PKR and OAS/RNase L, that have global effects on protein synthesis or mRNA stability.

Materials and methods

Cells, viruses and interferon

Vero and Hep2 cells, and their derivatives were grown as monolayers in 25 cm^2 or 75 cm^2 tissue culture flasks, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum or fetal bovine serum (growth medium) at 37 °C. When required, cells were treated with recombinant human interferon-α2a [rHuIFN-α (Roferon-A; Roche)] at 1000 units/ml. PIV5 strains CPI− and CPI+ (Baumgartner et al., 1982, 1987; Evermann et al., 1981) were grown and titrated under appropriate conditions in Vero cells.

CPI− and CPI+ infection and IFN treatment time course

Cell monolayers were infected with CPI− or CPI+ (the parental strain of CPI− that blocks IFN signaling; Chatziandreou et al., 2002) strains of PIV5 at an m.o.i. of 50 to 100 pfu/cell (or mock-infected). After an adsorption period of 1–2 h on a rocking platform at 37 °C, the virus inoculum (or growth medium, for mock infections) was removed and replaced with fresh maintenance medium (DMEM containing 2% newborn calf serum or fetal bovine serum). At 12 h p.i. the medium was either supplemented with rHuIFN-α or left untreated as a control. Cells were incubated for a further 6 or 12 h (as indicated in each experiment) and then harvested for protein analysis, as described below.

siRNA interference

Hep2 naïve cells and Hep2 cells that constitutively express the NPro protein of bovine viral diarrhea virus (Hep2/BVDV-NPro) were transfected with either a pool of siRNAs specific for human RNase L (SMART pool; Dharmacon Research, Lafayette, CO) or a siRNA targeting human PKR (siRNA PKR duplex; Dharmacon Research, Lafayette, CO). The final concentration of siRNA was 100 nM and the transfection reagent used was Oligofectamine (Invitrogen). At 30 h after transfection, the cells were infected with CPI− and treated or not treated with IFN for 6 h, as described above. Immunoblotting analysis (described below) was performed to measure the levels of RNase L, PKR and actin.

Immunoprecipitation, immunoblotting and immunofluorescence

The procedures for immunoblotting, immunofluorescence and immunoprecipitating radioactively labeled proteins have previously been described (Carlos et al., 2005; Randall and Dinwoodie, 1986). Antibodies used in these procedures included monoclonal antibodies (mAbs) to the NP, P, M and HN proteins of PIV5 (Randall et al., 1987) and a polyclonal antiserum to the P and V proteins of PIV5 (Carlos et al., 2005). MxA was detected with a polyclonal anti-MxA antibody (kindly provided by G. Kochs, University of Freiburg, Germany). The phosphorylated form of eIF-2α (eIF-2α-P) was detected with a rabbit anti-Phospho-eIF-2α (Ser51) antibody (Cell signalling). PKR was detected using a rabbit polyclonal anti-PKR (Santa Cruz Biotechnology) and RNase L was detected using a mouse monoclonal anti-RNase L antibody (Zymed Laboratories Inc.). Actin was detected using a mouse monoclonal anti-β-Actin antibody (Sigma).

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