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The orf virus OV20.0L gene product is involved in interferon resistance and inhibits an interferon-inducible, double-stranded RNA-dependent kinase

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SUMMARY

The parapoxvirus orf virus was resistant to type 1 (IFN-α) and type 2 (IFN-γ) interferons in cultures of ovine cells. The recently identified orf virus OV20.0L gene exhibits 31% predicted amino acid identity to the vaccinia virus E3L interferon-resistance gene, and is referred to as the (putative) orf virus interferon-resistance gene (OVIFNR). The objective of this study was to determine whether OVIFNR was involved in interferon resistance. Recombinant OVIFNR as a thioredoxin fusion protein (OVIFNR–Tx) inhibited the activation (by autophosphorylation) of an interferon-inducible, double-stranded (ds) RNA-dependent kinase (PKR) of sheep, which was shown to bind dsRNA (poly I:C). PKR in other species is involved in the inhibition of protein synthesis as part of the antiviral state in infected cells. Virus-infected cell lysates, but not control lysates, from cells grown in the presence of cytosine arabinoside also contained PKR inhibitory activity, which indicated that the inhibitory activity was associated with early viral gene expression. Significantly, the OVIFNR gene expressed in interferon-treated ovine fibroblasts protected the unrelated Semliki Forest virus from the antiviral effect of both type 1 and type 2 interferons. Taken together, the results indicate that the OVIFNR gene functions as an interferon-resistance gene, the product of which inhibits PKR in a similar way to the vaccinia virus E3L gene product.

INTRODUCTION

Poxviruses stimulate a vigorous immune response in their infected hosts. In spite of this, the viruses can replicate and induce lesions. The immune response to poxviruses is potentially lethal for the virus, as exemplified by the successful eradication of smallpox (caused by variola virus) in the human population by vaccination with the related, but less virulent, vaccinia virus. Recently, it has become apparent that the survival of poxviruses in the presence of an active immune response is caused in large part, if not solely, by the expression of virus virulence genes that interfere with host immune and inflammatory response effector molecules.[1–4] Many of these are viral orthologues of host cellular genes that have been acquired and modified by the viruses. The protein products of these genes, in general, target effector molecules of the early phase of the host antiviral inflammatory and immune response, including interferons, complement and the cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α).[1–5]

The importance of interferon (IFN) in protection against orthopoxviruses is underlined by the discovery of several viral proteins that either block IFNs directly by mimicking soluble IFN receptors and preventing them from docking to their cellular receptors, or that inhibit IFN-inducible proteins produced as part of the antiviral state.[4,6] Of particular importance for viral interference is the IFN-inducible, double-stranded (ds) RNA-dependent kinase (PKR) pathway.[7,8] PKR contributes to the antiviral state within cells by binding to viral dsRNA whereupon it is activated to phosphorylate itself and the α-chain of the translation initiation factor eIF-2. This inactivates eIF-2 and inhibits virus (and cell) protein translation, effectively blocking virus replication. The vaccinia virus genes E3L and K3L encode proteins that inhibit PKR by competing with the enzyme for dsRNA binding and acting as a decoy for eIF-2 respectively.[6,9,10]

The parapoxvirus orf virus is the prototype for a group of viruses that induce acute cutaneous lesions in different mammalian species, but that in all cases also infect humans.[11] Orf virus naturally infects sheep, goats and humans through broken or damaged skin, replicates in regenerating epidermal cells and induces pustular lesions that turn to scab. Virus is shed with the scab and there is no evidence of systemic spread. Orf virus will repeatedly infect previously exposed lambs and induce lesions. Recently, several putative immune-modulating virulence genes have been discovered within the orf virus genome. These include viral homologues of ovine vascular endothelial growth factor, interleukin-10 (IL-10) and a granulocyte–macrophage colony-stimulating factor (GM-CSF) inhibition gene.[11–13] An orf virus gene mapping to an open reading frame at position OV20.0L at the left terminus of the

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viral genome[14] has recently been isolated and cloned by A. Mercer, and exhibits 31% consensus amino acid sequence identity to the vaccinia virus E3L IFN-resistance gene product (unpublished observation). For this reason, the gene is referred to as the (putative) orf virus IFN-resistance gene (OVIFNR). Preliminary unpublished studies indicated that the orf virus was resistant to IFNs in tissue culture. The specific objectives of the present study were as follows.

(1) To confirm that orf virus is resistant to type 1 and type 2 IFNs in cell culture.

(2) To characterize ovine PKR and determine whether it is inhibited in orf virus-infected cells.

(3) To determine whether the OVIFNR gene product inhibits PKR and is associated with IFN resistance.

MATERIALS AND METHODS

Cells and virus

Fetal lamb muscle (FLM) primary cell cultures and the ST-6 ovine fibroblast cell line were developed and maintained as described previously.[15] HeLa cells were obtained from the ECACC (Salisbury, UK). All cells were cultured in Medium 199/10% fetal calf serum (FCS) (Gibco BRL, Paisley, UK). Orf virus strain NZ2[16] was passaged in FLM cells, purified and stored at −70° in aliquots. Semliki Forest virus (SFV, an RNA-cytopathic virus) was passaged in ST-6 cells.[15] Tissue culture infective dose of virus ($TCID_{50}$) values based upon the dilution of virus giving 50% cytopathic effect (CPE) were obtained for both viruses in appropriate cells used in the experiments.[15] For detection of early viral gene products, FLM or ST-6 cells were infected with orf virus at a multiplicity of infection (MOI) of ~20 $TCID_{50}$ viruses/cell in medium containing 40 μg/ml cytosine arabinoside. This dose was shown to prevent viral CPE in this study. Cell lysates were collected at 5 and 15–17 h later. Cytosine arabinoside inhibits poxviral DNA synthesis but not early viral gene expression.[17]

Cell lysates were prepared as described previously[18] in ice-cold lysis buffer (0·5% IGEPAL CA-630 (Sigma, Poole, UK), 0·1 M KCl, 0·001 M $Mg(C_2H_3O_2)_2$, 0·01 M HEPES pH 7.5 in water) containing protease inhibitors (Boehringer Complete®; Boehringer Mannheim, Lewes, UK). Protein estimation was by the BCA method (Pierce, Leiden, the Netherlands).

Interferon resistance assay

Dilutions of purified human IFN-α (Wellcome, Beckenham, UK) or recombinant ovine IFN-γ (rovIFN-γ)[19] in medium were added to ST-6 cells in six replicate wells of 48-well plates and incubated in a humidified incubator flushed with 5% CO_2 in air. Control wells received medium only. After 18 h, the cells were infected with 2000 $TCID_{50}$ NZ2 orf virus or 500 $TCID_{50}$ SFV/well and incubated for a further 48 h during which time cells with virus but no IFN exhibited 100% CPE, as determined visually by standard criteria.[15,19] As some dead and dying cells in the absence of virus can exhibit similarities to viral-induced CPE, a value of 10% or greater CPE was considered to represent genuine CPE. The mean CPE for each of the six replicate wells was determined as a measure of virus susceptibility (no CPE) or resistance (CPE) to the different dilutions of the IFNs.

PKR and PKR-inhibition assays

Cell lysates from FLM cells, incubated for 20 h in the presence of 200 U/ml human IFN-α, were used as a source of PKR.[7,9,18] Poly I:C (Pharmacia, Uppsala, Sweden) dsRNA was used to activate PKR and stimulate substrate phosphorylation detected by SDS–PAGE and autoradiography.[9,18] Kinase buffer consisted of 50 mM $MgCl_2$, 0·5 mM ATP and 37 kBq [γ^{32}-P]-ATP/10 μl (Amersham, Bucks, UK) in water. dsRNA was added to 75 μg control or IFN-treated cell lysates in microfuge tubes on ice to give final dsRNA concentrations of 0, 0·01, 0·1 and 1 μg/ml. Ten microlitres of kinase buffer was added to give a final volume of 20 μl/tube. The tubes were incubated at 30° for 15 min, and the phosphotransferase reaction terminated by the addition of SDS–PAGE sample buffer, containing 2-mecaptoethanol, and incubation in a boiling water bath for 4 min. Twenty microlitre samples from each tube were analysed by SDS–PAGE. Gels were stained with Coomassie brilliant blue, dried and phosphorylated proteins detected by autoradiography.[18]

The PKR inhibition assay involved the additional step of adding dilutions of orf virus-infected cell lysates, OVIFNR–thioredoxin (Tx) fusion protein (OVIFNR–Tx) or control Tx protein to the tubes on ice followed by the dsRNA dilutions. After 5 min, the IFN-treated cell lysates were added, the tubes were warmed to 30° and the procedure followed as described above. Seventy-five micrograms of the orf virus-infected cell lysates and 0·1–5 μg of the OVIFNR–Tx or control Tx proteins were used in these assays, based on dose–response studies.

Ovine PKR was detected in IFN-treated or control cell lysates by SDS–PAGE and Western blot using the cross-species PKR-specific rabbit polyclonal M-515 antibody (sc-1702, Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL technique (Amersham Little Chalfont, UK).

Affinity removal of dsRNA-binding proteins

The method used for affinity removal of dsRNA-binding proteins was based upon that described by Silverman & Krause.[19] Ten microlitres of washed poly (I:C)-agarose suspension (Pharmacia) in incubation buffer, and lysates (100 μg) of control or IFN-treated FLM cells were added to microfuge tubes on ice. Reaction volumes were made up to 25 μl with incubation buffer (10 mM HEPES pH 7·5, 50 mM KCl, 1·5 mM Mg $(C_2H_3O_2)_2$, 7 mM β-mercaptoethanol and 10% v/v glycerol) and incubated for 1 h at 0° with occasional gentle vortexing. Supernates were obtained after centrifugation, and poly (I:C)-bound proteins obtained after washing the agarose complexes in lysis buffer (see above) containing proteinase inhibitors. The presence or absence of PKR was determined both by PKR assay and also by Coomassie brilliant blue staining of SDS–PAGE gels.

Expression of the OVIFNR cDNA and purification of the OVIFNR–Tx fusion protein

The OVIFNR cDNA representing the coding region of the gene in the NZ2 orf virus strain was cloned into the pTrxFus expression vector (Invitrogen BV, Leek, the Netherlands) and the OVIFNR protein was expressed in *Escherichia coli* as a thioredoxin fusion protein OVIFNR–Tx. OVIFNR–Tx and control Tx proteins were extracted from bacterial cells and purified.

For transient expression of OVIFNR protein in FLM and

ST-6 cells, the cDNA was cloned into the pEE14 mammalian expression vector (Celltech, Slough, UK)[20] which was used to transfect (DNA-mediated transfer) cells in culture using the Qiagen (Crawley, UK) superfect® cationic lipid reagent (lipid:DNA of 6:1, for 4 h). Transfection efficiency was monitored by transfecting the cells with a plasmid containing a β-galactosidase (β-gal) cDNA and staining for β-gal in cells using X-gal substrate.

OVIFNR IFN-resistance assay

The OVIFNR gene product was tested for its ability to protect SFV from the antiviral effect of type 1 and type 2 IFNs following DNA-mediated transfer into ovine ST-6 and FLM fibroblasts. Following DNA transfection with OVIFNR–pEE14 or pEE14 control plasmid DNA, cells in 48-well plates were incubated for 18 h with dilutions of IFN-α or IFN-γ. Five hundred $TCID_{50}$ of SFV was added to appropriate wells and the CPE measured daily for 4 days. One-hundred per cent CPE was observed in both cell types within 48 h of SFV infection in the absence of IFN and transfected DNA in all experiments. Other control wells contained cells and transfected DNA only; IFN only; virus and IFN dilutions; transfected DNA and IFN dilutions; transfected DNA and virus; and medium only.

Statistical analysis

Where appropriate, the Student's *t*-test was applied to data normalized by $\log_{10}$ transformation.

RESULTS

Orf virus is resistant to IFNs in culture

Table 1 shows the effect of different concentrations of IFN-α and IFN-γ on either orf virus or control SFV-induced CPE measured at 48 h after infection when ST-6 cells with either virus alone showed 100% CPE. IFN-α at doses of 10 U/ml and above protected ST-6 cells from SFV-induced CPE, whereas IFN-γ was protective at 50, 100 and 200 U/ml. IFN-α did not protect the cells from orf virus-induced CPE at any of the concentrations used, whereas IFN-γ demonstrated some protection at the relatively high doses of 100 and 200 U/ml. This partial protection was transient, however, as by 4 days after infection CPEs in these wells were between 95 and 100%. Infective virus was recovered from orf virus-infected cells in the presence of IFN (as detected by the ability of cell lysates plus supernates to infect FLM cells). Similar results were obtained using IFN-treated and untreated, orf virus-infected FLM cells.

Table 1. Orf virus resistance to interferons

Sample	Mean CPE (%)	
	Orf virus	Semliki Forest virus
Medium control	0	0
Virus	100	100
Virus + 200 U/ml IFN-α	100	< 10
Virus + 100 U/ml IFN-α	100	< 10
Virus + 50 U/ml IFN-α	100	< 10
Virus + 20 U/ml IFN-α	100	< 10
Virus + 10 U/ml IFN-α	100	20
Virus + 1 U/ml IFN-α	100	50
Virus + 200 U/ml IFN-γ	62·7	< 10
Virus + 100 U/ml IFN-γ	70·8	< 10
Virus + 50 U/ml IFN-γ	85	< 10
Virus + 20 U/ml IFN-γ	90	23·3
Virus + 10 U/ml IFN-γ	100	38·3
Virus + 1 U/ml IFN-γ	100	90·5

ST-6 cells were treated with interferons (IFNs) for 18 h then infected with orf virus (NZ-2) or Semliki Forest virus. Cytopathic effect (% CPE) was measured at 48 h after infection. A CPE of 10% or greater in wells containing IFN indicated virus resistance to IFN.

Identification of ovine PKR

PKR, in mouse and humans, is detected in cell lysates as a 65/66 (mouse) or 68/69 (human) kDa IFN-inducible, dsRNA-activated phosphorylated protein by SDS–PAGE and autoradiography.[9,19] Figure 1(a) shows a 65 kDa, dsRNA-acti-

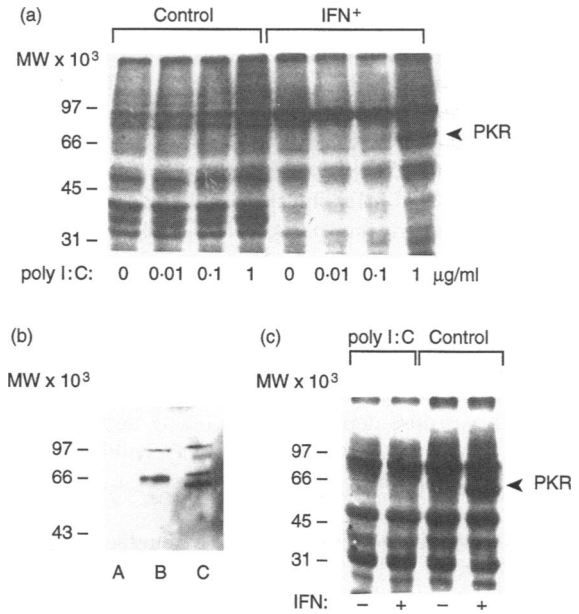

Figure 1. Detection of the ovine interferon-inducible, dsRNA-dependent kinase (PKR). All lanes in any one of the gel analyses shown received the same amount of cell lysate protein. (a) Untreated (control) and interferon-α-treated (IFN^+) FLM cell lysates were subjected to the *in vitro* kinase assay followed by SDS–PAGE (reducing conditions) and autoradiography. The 65 kDa phosphoprotein in the IFN-treated cell lysates in the presence of dsRNA (polyI:C) is the putative ovine PKR. (b) Western blot, with the pan PKR-specific M-515 antibody, of untreated (lane A) and IFN-treated (IFN+, lane B) FLM cell lysates used in (a) after SDS–PAGE (reducing conditions) and transfer to nitrocellulose. IFN-α-treated HeLa cell lysate (lane C) was included as a positive control for the human PKR (a doublet in this case). Comparing lanes A and B shows that the ovine PKR (65 kDa) was induced by IFN. The higher molecular-weight band in lanes B and C is an uncharacterized band recognized by the M-515 antibody in some cell types. (c) Removal of PKR from IFN-treated (+) FLM cell lysates by affinity binding to dsRNA (polyI:C-agarose = polyI:C) but not control agarose (control). Following the binding reaction, the agarose was removed by centrifugation and the lysates analysed for PKR by *in vitro* kinase assay, SDS–PAGE (reducing conditions) and autoradiography.

vated phosphoprotein contained in IFN-treated but not control FLM cell lysates. This was the only IFN-induced, dsRNA-activated phosphoprotein reproducibly detected in the kinase assay ($n=6$). Confirmation that this was the ovine equivalent of PKR was obtained by immunoreactivity of the 65 kDa protein with the pan-PKR-specific M-515 antibody by Western blot (Fig. 1(b)).

The ovine PKR was removed from IFN-treated cell lysates by affinity binding to poly (I:C)-agarose, as determined by Coomassie brilliant blue-stained SDS–PAGE gels (not shown) and by PKR assay (Fig. 1(c)). IFN-treated cell lysates incubated with agarose alone retained PKR, and PKR phosphorylation was detected. Attempts to detect PKR bound to poly I:C-agarose were unsuccessful.

Orf virus-infected cell lysates contain PKR inhibitory activity

PKR inhibitory activity was observed in lysates of FLM cells infected with orf virus at a MOI of 20 for 5 h (no CPE) and to a lesser extent in cells infected at a MOI of 1 for 15–20 h (10–50% CPE in three experiments). Figure 2 shows that cell lysates from FLM cells infected for 5 h with orf virus, in the presence of cytosine arabinoside, diminished PKR phosphorylation. This indicated that the inhibitory activity was the product of early viral gene expression. Inhibitory activity was also detected in two of three lysates from cells treated in the same way (MOI of 20, 40 μg/ml cytosine arabinoside) but collected at 15–20 h after infection. This suggested that the inhibitory activity was not particularly labile.

Recombinant OVIFNR–Tx protein inhibits PKR

The recombinant OVIFNR–Tx and control Tx proteins are shown in Fig. 3(a). Figure 3(b) shows that OVIFNR–Tx, but not control Tx, inhibited PKR phosphorylation in the kinase assay. This was dose dependent with 0·5 μg/ml OVIFNR–Tx the lowest concentration that inhibited any visible evidence of PKR phosphorylation.

The OVIFNR gene product protects SFV from the antiviral effect of IFN

Transient expression of the OVIFNR cDNA in ST-6 cells or FLM cells was associated with protection of SFV from IFN-α

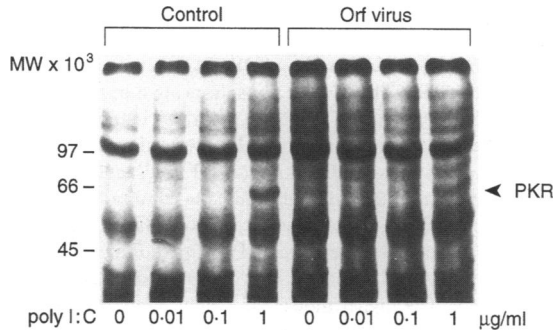

Figure 2. Inhibition of PKR phosphorylation by lysates from orf virus-infected cells (Orf virus) but not control cells (control) grown in the presence of cytosine arabinoside. Cytosine arabinoside inhibits early but not late viral gene expression. *In vitro* kinase assay, SDS–PAGE and autoradiography. All lanes of the gel contained the same quantity of protein (75 μg).

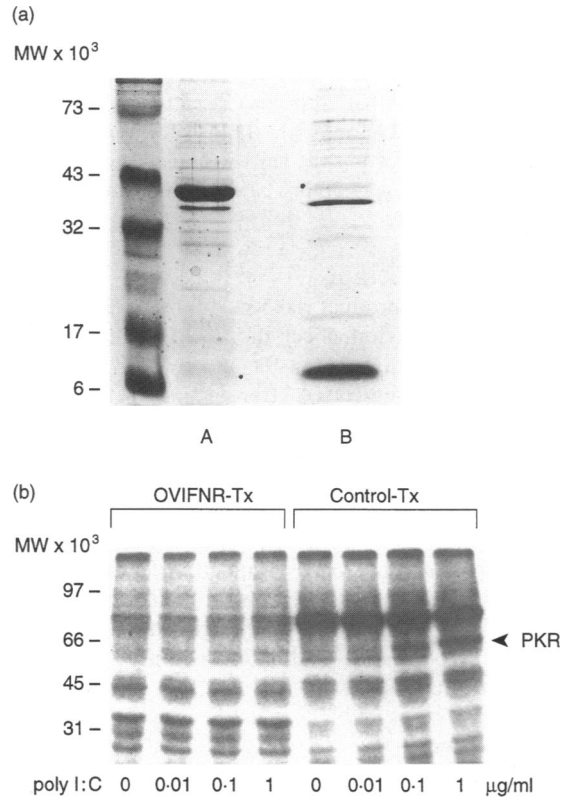

Figure 3. The OVIFNR–Tx fusion protein inhibits PKR. (a) OVIFNR–Tx and control Tx proteins after purification. Coomassie brilliant blue–stained SDS–PAGE gel (reducing conditions). Lane A, OVIFNR–Tx (36 kDa), lane B, control Tx (12 kDa). (b) Inhibition of PKR phosphorylation by OVIFNR–Tx but not by control Tx. The concentration of each protein added to lanes of the gel was 2 μg.

over a range of IFN concentrations. The results of an experiment in ST-6 cells is shown in Fig. 4 (representative of three in total). SFV-induced CPE was significantly more extensive in wells with cells transfected with OVIFNR cDNA and containing IFN compared to the CPE in wells with control plasmid cDNA and equivalent concentrations of IFN ($P<0·02$ for all IFN concentrations). Increasing the concentration of IFN decreased the SFV-induced CPE, indicating a possible competition between IFN-induced effector molecules and the OVIFNR protein for a common target molecule or biochemical pathway. Transfection efficiency in FLM and ST-6 cells was assessed by β-gal cDNA expression and measuring the frequency of X-gal-staining cells, and was within the range of 20–55% ($n=4$). In a similar set of experiments in ST-6 cells, transient expression of OVIFNR protected SFV from the antiviral effect of IFN-γ used at 5, 10 or 20 U/ml ($n=3$).

DISCUSSION

In this study, orf virus resistance to type 1 and type 2 IFNs, over a range of IFN concentrations, was demonstrated *in vitro*. The recent discovery of the OVIFNR gene in the left terminus of the orf virus genome with homology to the vaccinia virus E3L gene raised the possibility that the OVIFNR gene product may be responsible, at least in part, for orf virus resistance to IFNs. The vaccinia virus E3L protein has been

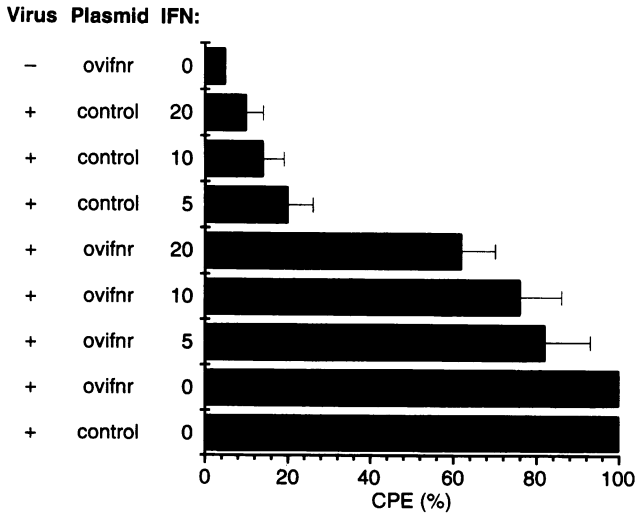

Figure 4. OVIFNR-induced protection of Semliki Forest virus (SFV) (virus) from the antiviral effect of interferon-α (IFN). ST-6 cells were transfected with control PEE14 plasmid (control) or OVIFNR–PEE14 plasmid (ovifnr) prior to treatment with 0–20 U/ml IFN. Forty-eight h after infection with SFV, the cultures were scored for cytopathic effect (% CPE). IFN protected the cells from SFV-induced CPE, but this was inhibited in cells transiently expressing OVIFNR protein.

shown to compete with the IFN-induced serine/threonine kinase PKR for binding to dsRNA produced during virus infection of cells. This prevents dsRNA activation of PKR by autophosphorylation and hence PKR-mediated inactivation (by phosphorylation) of the translation initiation factor eIF-2α.[6–10] As a result, virus (and cell) protein translation proceeds and the antiviral effect of interferon is neutralized.

Orf virus-infected cell lysates contained an activity that diminished ovine PKR phosphorylation, an activity shared by purified OVIFNR–Tx protein. In addition, the OVIFNR gene product transiently expressed in ovine cells rescued SFV from the antiviral effect of both type 1 and type 2 IFNs. PKR inhibitory activity was detected in orf virus cell lysates from cultures containing cytosine arabinoside, indicating that the activity was associated with early viral gene expression and a natural consequence of orf virus infection of target cells. Although this activity was not mapped directly to the OVIFNR gene product, a separate study has shown that the OVIFNR gene is transcribed as an early viral gene. (C. McInnes, unpublished observation) Ovine PKR was removed from lysates by poly (I:C)-agarose, demonstrating that the ovine enzyme, like PKR in mouse and humans is a dsRNA-binding protein. The inability to detect phosphorylated PKR after elution from poly (I:C)-agarose may have been a result of the high concentration of dsRNA bound to the agarose, which has been shown to inhibit PKR autophosphorylation.[18] OVIFNR–Tx is also a dsRNA-binding protein that does not bind to ssRNA, ssDNA or dsDNA (C. McInnes, unpublished, observation). Taken together, the above results indicate that the OVIFNR protein is biochemically similar to the vaccinia virus E3L protein, and suggest a similar mechanism of IFN resistance, via inhibition of PKR, by competitive binding of dsRNA.

PKR is an important enzyme targeted by various viruses exhibiting resistance to IFNs. For example, reovirus σ3 protein inactivates PKR by binding dsRNA[21] in an analogous manner to vaccinia virus E3L and orf virus OVIFNR proteins. However, adenovirus has evolved a different strategy and expresses small RNAs (VA RNA_I and VA RNA_{II}) that bind to and inhibit PKR.[22] The importance of PKR in host protection against viruses is best exemplified by vaccinia virus, which has two genes with products that target the enzyme. E3L competes with PKR for dsRNA binding whereas K3L has homology to eIF-2 and acts as a decoy for the substrate, thus inactivating PKR.[6] PKR may be involved in different aspects of the IFN-induced antiviral state, and not just inactivation of eIF-2. PKR regulation of both IFN production and apoptosis within cells has been reported.[23] However, these aspects of PKR function are not fully characterized and their relative importance compared to eIF-2 inhibition in the IFN-induced antiviral state is not known. Furthermore, PKR is not the only IFN-induced protein involved in the antiviral state. The 2′5′ adenocyclase pathway is also dependent upon dsRNA and induces an RNase that degrades mRNAs and rRNAs.[7] This pathway is not induced in vaccinia virus-infected cells, possibly because of inhibition by viral ATPase and phosphatase.[7] In mice, IFN-γ inhibits vaccinia virus DNA synthesis in infected macrophages, an activity associated with nitric oxide production.[24] It is not known if these IFN-inducible pathways operate in orf virus-infected ovine cells but, if they do, they are ineffective in preventing viral CPE and replication.

The discovery, in this study, that the OVIFNR gene in orf virus is functional *in vitro* confers on the virus the potential for IFN resistance *in vivo*. Orf virus infection differs from vaccinia virus infection in susceptible hosts by being an acute localized infection with no evidence of systemic spread or chronic infection. Type 1 and type 2 IFNs are detected in lymph draining the site of viral reinfections and the IFN genes are transcribed in the skin during primary infections and reinfections.[25–28] Sheep treated with cyclosporin-A during a reinfection developed severe primary-type lesions. This was associated with inhibition of IFN-γ and interleukin-2 gene transcription in the skin of cyclosporin-A-treated and reinfected animals.[28] We speculate that IFNs are important in host protection against parapoxvirus infection, as they are in protection against orthopoxviruses, and that the orf virus OVIFNR gene product may protect the virus from the antiviral effect of IFNs for long enough to allow at least some viral replication. This hypothesis will be tested using recombinant orf viruses lacking a functional OVIFNR gene.

ACKNOWLEDGMENTS

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