Evidence that avian reovirus σA protein is an inhibitor of the double-stranded RNA-dependent protein kinase

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Received 25 November 2002
Accepted 6 February 2003

The results of a previous study demonstrated that avian reovirus is highly resistant to the antiviral effects of interferon and suggested that the double-stranded RNA (dsRNA)-binding σA protein might play an important role in that resistance. To gather more evidence on the interferon-inhibitory activity of σA protein, its gene was cloned into the prokaryotic maltose-binding protein (MBP) gene fusion vector pMalC and into the recombinant vaccinia virus WRS2. The two recombinant σA proteins displayed a dsRNA-binding affinity similar to that of σA protein synthesized in avian reovirus-infected cells. Interestingly, MBP–σA, but not MBP, was able to relieve the translation-inhibitory activity of dsRNA in reticulocyte lysates by blocking the activation of endogenous dsRNA-dependent enzymes. In addition, transient expression of σA protein in HeLa cells rescued gene expression of a vaccinia virus mutant lacking the E3L gene, and insertion of the σA-encoding gene into vaccinia virus conferred protection for the virus against interferon in chicken cells. Further studies demonstrated that expression of recombinant σA in mammalian cells interfered with dsRNA-dependent protein kinase (PKR) function. From these results we conclude that σA is capable of reversing the interferon-induced antiviral state by down-regulating PKR activity in a manner similar to other virus-encoded dsRNA-binding proteins.

INTRODUCTION

The interaction of extracellular interferon (IFN) with species-specific cell-surface receptors initiates a signal transduction pathway that leads to increased expression of more than 30 different proteins, some of which are thought to play an important role in fighting viral infections (for reviews, see Foster, 1997; Goodbourn *et al.*, 2000; Stark *et al.*, 1998). Among the best characterized are the 2′,5′-oligoadenylate synthetase system (2-5A synthetase) and the double-stranded RNA (dsRNA)-activated protein kinase (PKR), which are activated by dsRNA and play key roles in regulating intracellular protein synthesis (reviewed in Clemens, 1997; Player & Torrence, 1998).

IFN induces several different forms of 2-5A synthetase, which, on interaction with dsRNA, catalyse the conversion of ATP into short oligonucleotides of the general structure ppp(A2′p5′)nA. These oligonucleotides bind and activate a latent endoribonuclease L (RNase L), which catalyses the indiscriminate degradation of RNAs, thereby leading to a general inhibition of intracellular protein synthesis (reviewed in Rebouillat & Hovanessian, 1999). RNase L has also been shown to cleave ribosomal RNAs (rRNAs) in a site-specific manner (Iordanov *et al.*, 2000; Rivas *et al.*, 1998; Wreschner *et al.*, 1981). On the other hand, activation of PKR by dsRNA interaction leads to phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2, giving rise to inhibition of protein synthesis by preventing the recycling of initiation factors (reviewed in Clemens, 1997; Clemens & Elia, 1997). Activation of these two IFN-induced antiviral systems has also been shown to trigger apoptosis in a way that is independent of the particular means of achieving translational inhibition (Iordanov *et al.*, 2001; Castelli *et al.*, 1997; Diaz-Guerra *et al.*, 1997; Lee & Esteban, 1994).

Avian reoviruses are members of the *Orthoreovirus* genus, one of nine genera of the *Reoviridae* family. They are non-enveloped viruses that replicate in the cytoplasm of infected cells and contain ten dsRNA genome segments enclosed in a double protein capsid shell 70–80 nm in diameter (reviewed in Robertson & Wilcox, 1986). The avian reovirus genome encodes at least ten structural proteins and four non-structural proteins, although very little is known about

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the functions of most of these proteins (Bodelon *et al.*, 2001; Varela *et al.*, 1996).

A previous study showed that four avian reovirus strains, including S1133, are resistant to the antiviral action of a natural chicken IFN produced in embryonated eggs (Ellis *et al.*, 1983). A recent study, performed in our laboratory, revealed that exposure of chicken embryo fibroblasts (CEFs) to a recombinant chicken interferon (rcIFN) induces a strong intracellular antiviral state sufficient to inhibit the replication of vesicular stomatitis virus and vaccinia virus but not the replication of avian reovirus S1133. In the same study we also showed that the translation-inhibitory activity of dsRNA in reticulocyte lysates can be relieved by extracts of avian reovirus-infected cells, suggesting the presence of a protranslational factor in these extracts. Further *in vitro* translation experiments indirectly suggested that this factor is avian reovirus σA protein (Martínez-Costas *et al.*, 2000). Protein σA is a minor component of the virus inner capsid that binds dsRNA very tightly in a sequence-independent manner (Martinez-Costas *et al.*, 1997, 2000; Yin *et al.*, 2000). However, no consensus dsRNA-binding motifs have been found in the amino acid sequence of σA protein, as found in the sequences of the dsRNA-binding proteins NS1 of influenza virus and VP6 of bluetongue virus (Hatada & Fukuda, 1992; Stauber *et al.*, 1997).

In this study we have performed experiments to characterize the IFN-inhibitory activity of avian reovirus σA protein. Our results suggest that σA is a key factor in the IFN-resistant phenotype displayed by avian reovirus because of its ability to down-regulate PKR function.

METHODS

Cells and viruses. Primary cultures of CEFs were prepared from 9- to 10-day-old chicken embryos and grown in monolayers in medium 199 supplemented with 10 % tryptose phosphate broth and 5 % calf serum. African green monkey kidney BSC-40 and human HeLa cell lines were grown in monolayers in medium 199 supplemented with 10 % foetal bovine serum (FBS). Strain S1133 of avian reovirus was grown in semiconfluent monolayers of primary CEFs, as previously described (Grande & Benavente, 2000). Vaccinia viruses were propagated in BSC-40 cells, as previously described (Lee & Esteban, 1994). In this study we used wild-type vaccinia virus WR strain and the following recombinant vaccinia viruses: WR68K, which expresses a human recombinant IPTG-inducible PKR (Lee & Esteban, 1994); WRLuc, which constitutively expresses a recombinant firefly luciferase protein (Gherardi *et al.*, 1999); WRE3L^{-}, which lacks the E3L gene (Beattie *et al.*, 1995); and WRS2, which was generated in our laboratory as follows. Semi-confluent monolayer cultures of BSC-40 cells were infected with 0·01 p.f.u. per cell of WR and after 1 h adsorption at 37 °C, the inoculum was removed and the cells were lipofected with 2·5 μg per 10^{6} cells of the vaccinia virus insertion plasmid pHLZ/S2 (see below). The cells were subsequently incubated for 5 h at 37 °C, then the transfection medium was removed and replaced by DMEM supplemented with 3 % FBS. At 48 h post-transfection, the cells were harvested, subjected to three cycles of freezing and thawing and sonicated. Serial dilutions of the cell extracts were used to infect fresh BSC-40 cell monolayers and after 1 h adsorption at 37 °C, the inoculum was removed and replaced by DMEM supplemented with 2 % FBS and 1 % agar. Three days later, the agar was covered with DMEM supplemented with 1 % agar and 0·03 % X-Gal and the cells were incubated for 12–24 h. Blue plaques were selected and homogeneous recombinant virus was isolated by three more rounds of plaque purification. WRS2 contains the σA-encoding gene under the control of the synthetic early/late PE/L promoter. The correct orientation of the S2 insert was confirmed by nucleotide sequencing. Virus titres were determined by plaque assay, as previously described (Martínez-Costas *et al.*, 2000).

Plasmids. To generate a plasmid expressing a maltose-binding protein (MBP)-tagged σA fusion protein, total RNA from avian reovirus-infected cells was amplified by RT-PCR with the forward primer 5′-GCGGGATCCACGATGGCGCGTG-3′ (*Bam*HI site underlined) and the reverse primer 5′-GCGAAGCTTGCGTACGACC-CTACGC-3′ (*Hin*dIII site underlined). The resulting cDNA was digested and cloned into the *Bam*HI and *Hin*dIII sites of pMalC (New England Biolabs) and the resulting recombinant plasmid, pMalCS2, was introduced into *E. coli* strain BL21. The correct orientation of the insert was confirmed by nucleotide sequencing.

Plasmid pPR15 (containing the luciferase reporter gene under the control of the vaccinia virus p4b late promoter), plasmid pPR35 (designed for IPTG-inducible expression of genes) and recombinant plasmid pPR35E3L have all been described previously (Diaz-Guerra *et al.*, 1997; Rodriguez & Smith, 1990). For the generation of the pPR35S2 plasmid and the recombinant vaccinia virus insertion plasmid pHLZ/S2, the S2 avian reovirus gene was excised from pMALCS2 plasmid by *Hin*dIII digestion and after filling the ends with Klenow DNA polymerase, the insert was digested again with *Bam*HI. The resulting DNA fragment was then inserted into the *Bam*HI and *Sma*I sites of the vaccinia virus insertion plasmid pPR35 and into the *Bgl*II and *Sma*I sites of the vaccinia virus insertion plasmid pHLZ (Rodriguez & Smith, 1990).

Bacterial expression, protein purification and antibody generation. For expression of MBP and MBP–σA, cultures of pMalC- and pMalCS2-transformed BL21 bacteria were grown in LB medium supplemented with 0·2 % glucose and 100 μg ampicillin ml^{-1} up to an optical density of 0·6 at 600 nm. The cells were then induced with 1 mM IPTG, incubated for 2 h at 37 °C and finally lysed by sonication in a buffer containing 0·25 % Tween 20, 1 mM DTT, 200 mM NaCl, 20 mM Tris/HCl, pH 7·5 and 1 mg lysozyme ml^{-1}. The resulting extracts were clarified by centrifugation and MBP and MBP–σA were purified from the supernatants using amylose–agarose columns, as described by the manufacturer (New England Biolabs). When indicated, protease factor Xa (New England Biolabs) was added to the MBP–σA-containing sample at a w/w ratio of 1 % in column buffer (20 mM Tris/HCl, pH 7·4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) and incubated overnight at 4 °C. Protein σA was subsequently isolated from the mixture by Q-Sepharose chromatography performed in a buffer containing 20 mM Tris/HCl, pH 7·5, and 50 mM NaCl. Alternatively, supernatant extracts were subjected to poly(I:C)–agarose chromatography (Amersham Bioscience), as previously described (Martínez-Costas *et al.*, 2000).

Preparation of rabbit polyclonal antibodies against both gel-purified and native σA was carried out as described previously (Bodelon *et al.*, 2001).

Interferon treatment, infections, *in vitro* translation, protein analysis, detection of apoptosis and analysis of rRNA integrity. Treatment of CEFs with rcIFN, infection of cell monolayers, *in vitro* translation, protein radiolabelling and SDS-PAGE analysis have all been described previously (Martínez-Costas *et al.*, 2000). Densitometric analysis of viral protein bands was performed using a FluorS MultiImager system and Quantity One software (Bio-Rad).

Immunoprecipitation, immunoblotting and affinity chromatography methods have all been described previously (Bodelon *et al.*, 2001; Martínez-Costas *et al.*, 2000). Detection of oligonucleosomal DNA fragments, estimation of the amount of cytoplasmic histone-associated DNA and analysis of the integrity of 18S and 28S rRNAs have also been described (Labrada *et al.*, 2002).

Transient transfection of HeLa cells. Semiconfluent HeLa cell monolayers grown in 12-well plates were infected with 2·5 p.f.u. WRE3L$^-$ per cell and transfected 1 h later with 0·2 μg pPR15 per well plus the indicated amounts of pPR35-derived plasmids, using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were then induced or not with 1·5 mM IPTG and lysed 24 h later. Luciferase activity in cell extracts was determined with a luminometer as previously described (Brasier *et al.*, 1989).

RESULTS AND DISCUSSION

Bacterial expression of σA protein, purification and generation of polyclonal antibodies

As a first step to raise polyclonal antibodies against avian reovirus σA protein, the avian reovirus S2 gene was cloned into the MBP gene fusion vector pMalC and the resulting recombinant plasmid, pMalCS2, was used to transform BL21 *E. coli* cells. A comparative electrophoretic analysis of the proteins present in extracts of bacteria that had been transformed with plasmids pMalC or pMalCS2 (Fig. 1A) showed that both MBP (Fig. 1A, lane 3) and MBP–σA (Fig. 1A, lane 4) were expressed in IPTG-induced cells, but

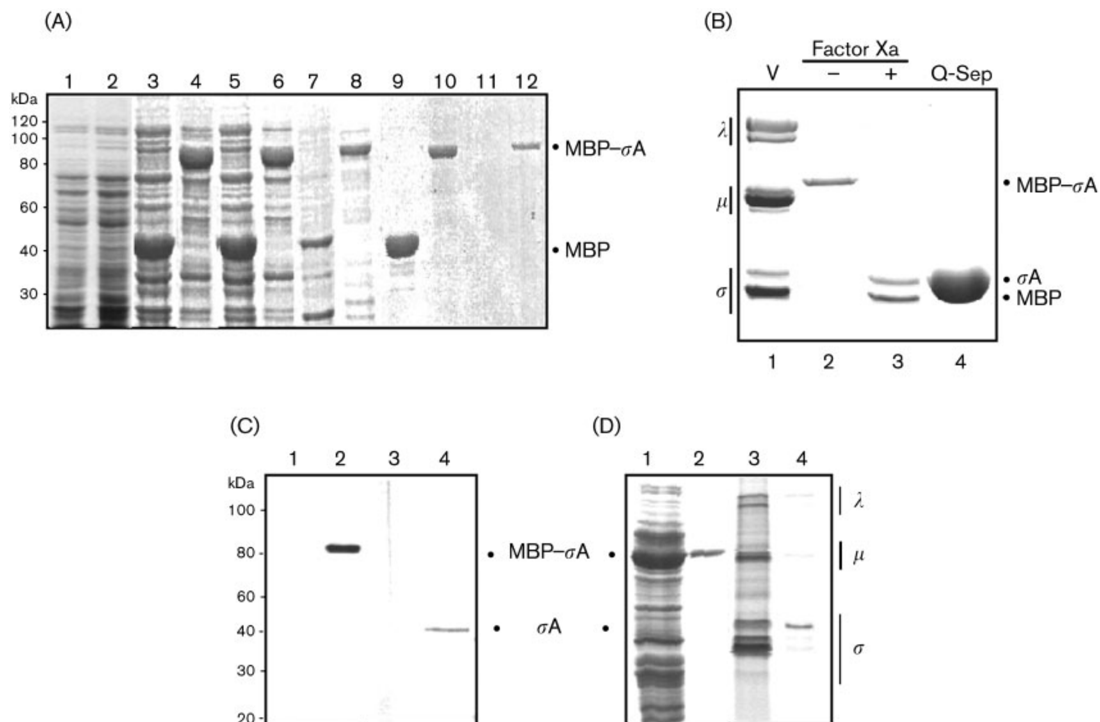

Fig. 1. Expression of σA protein in bacterial cells and characterization of anti-σA antibodies. (A) Cell extracts prepared from uninduced (lanes 1 and 2) or IPTG-induced bacteria transformed with plasmids pMalC (lane 3) or pMalCS2 (lane 4) were analysed by 12 % SDS-PAGE and stained with Coomassie blue. In the same gel, supernatant (lanes 5 and 6) and pelleted fractions (lanes 7 and 8) resulting from centrifugation of these extracts were also analysed, as well as proteins of the supernatant fractions that were retained on amylose–agarose (lanes 9 and 10) or on poly(I : C)–agarose beads (lanes 11 and 12). Positions of protein markers are indicated on the left and positions of MBP and MBP–σA on the right. (B) Electrophoretic analysis of reovirions (lane 1), amylose–agarose-purified MBP–σA (lane 2), factor Xa-digested MBP–σA (lane 3) and the fraction of Xa-digested MBP–σA that was not retained on a Q-Sepharose column (lane 4). Positions of the three size classes of avian reovirus polypeptides are indicated on the left and positions of σA, MBP and MBP–σA on the right. (C) Immunoblot analysis, with polyclonal antibodies against gel-purified σA, of extracts of MBP–σA-transformed bacteria either uninduced (lane 1) or after IPTG induction (lane 2) and of extracts of mock-infected (lane 3) and S1133-infected (lane 4) CEFs. Positions of protein markers are indicated on the left and positions of σA and MBP–σA on the right. (D) ^{35}S-labelled extracts of IPTG-induced, MBP–σA-transformed bacteria (lanes 1 and 2) and S1133-infected CEFs (lanes 3 and 4) were analysed by SDS-PAGE and autoradiography, either before (lanes 1 and 3) or after (lanes 2 and 4) immunoprecipitation with antiserum raised against native σA. Positions of the three size classes of avian reovirus polypeptides are indicated on the right and positions of σA and MBP–σA on the left.

not in the corresponding uninduced bacteria (Fig. 1A, lanes 1 and 2). Furthermore, when the extracts of induced bacteria were centrifuged, larger amounts of both MBP and MBP–σA appeared in the supernatant fractions (Fig. 1A, lanes 5 and 6) than in the pellet fractions (Fig. 1A, lanes 7 and 8), revealing that the two proteins were expressed mainly in soluble form. The identity of MBP–σA was initially assessed by affinity chromatography; protein MBP–σA was specifically retained on both amylose– and poly(I : C)–agarose columns (Fig. 1A, lanes 10 and 12), whereas MBP was retained on the former but not on the latter column (Fig. 1A, lanes 9 and 11). The identity of MBP–σA was further confirmed by immunological assays (see below).

Digestion of MBP–σA (Fig. 1B, lane 2) with protease factor Xa yielded two polypeptides (lane 3); the retarded polypeptide was identified as recombinant σA (rσA) because it comigrated with the σA protein of avian reovirions (Fig. 1B, lane 1) and because of its ability to bind dsRNA, but not amylose (data not shown). The rσA protein was subsequently isolated by ion exchange chromatography, using a Q-Sepharose column (Fig. 1B, lane 4).

Both native and gel-purified rσA were used as immunogens for polyclonal antibody production. The proteins were subcutaneously injected into rabbits and 2 weeks after the second boost antisera were collected and tested. A subsequent Western blot analysis (Fig. 1C) showed that while antiserum raised against gel-purified σA did not recognize any protein in extracts of either uninduced bacteria (Fig. 1C, lane 1) or uninfected CEFs (Fig. 1C, lane 3), it recognized both MBP–σA in extracts of IPTG-induced pMalCS2-transformed bacteria (Fig. 1C, lane 2) and naturally occurring σA protein present in extracts of avian reovirus-infected cells (Fig. 1C, lane 4). On the other hand, while the antiserum raised against native rσA was able to immunoprecipitate MBP–σA (Fig. 1D, lane 2) and S1133 σA (Fig. 1D, lane 4) from ^{35}S-labelled cell extracts and purified virions (Fig. 1D, lanes 1 and 3), it did not immunoprecipitate any protein from extracts of either uninduced bacteria or uninfected CEFs (data not shown).

Protein σA abolishes the inhibition of translation by dsRNA *in vitro*

The results of a previous study indirectly suggested that σA protein is able to prevent the activation of the dsRNA-dependent enzymes in reticulocyte lysates (Martínez-Costas *et al.*, 2000). To obtain direct evidence for the protranslational activity of this protein, we compared the capability of MBP and MBP–σA to relieve the translation-inhibitory activity of dsRNA in reticulocyte lysates (Fig. 2). The results showed that, while MBP and MBP–σA did not inhibit exogenous tobacco mosaic virus (TMV) mRNA translation (Fig. 2, compare lanes 3 and 4 with lane 2), dsRNA induced a drastic inhibition of TMV protein synthesis (Fig. 2, compare lane 5 with lane 2). Interestingly, whereas the inhibitory activity of dsRNA remained intact after preincubation with MBP (Fig. 2, lane 6), it was completely abolished after preincubation with MBP–σA (lane 7). This result indicates that σA is able to reverse the translation-inhibitory activity of dsRNA, probably because of its ability to sequester dsRNA from the dsRNA-dependent enzymes. To confirm this hypothesis, we next investigated how changes in the order of addition of dsRNA and MBP–σA to reticulocyte lysates affected the translational efficiency of the reticulocyte lysate. Compared with the standard condition (Fig. 2, lane 7), inhibition of translation was observed when the two compounds were added together without preincubation (Fig. 2, lane 8) and this was even more pronounced when MBP–σA was added 5 min later than dsRNA (Fig. 2, lane 9). A similar translational rescue was also observed when dsRNA was preincubated with Xa-excised σA instead of MBP–σA (results not shown).

Together, these findings demonstrate that MBP–σA exerts its protranslational activity by blocking the activation of endogenous dsRNA-dependent enzymes, rather than by inhibiting their activities. Our results also provide direct evidence that the previously proposed protranslational factor present in extracts of avian reovirus-infected cells is indeed σA protein (Martínez-Costas *et al.*, 2000).

Transient expression of rσA rescues WRE3L$^-$ gene expression in HeLa cells

As a first approach to document the capacity of rσA to reverse the antiviral activity of IFN *in vivo*, we investigated

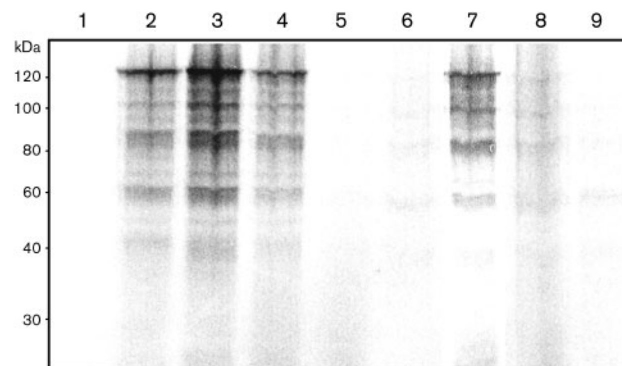

Fig. 2. MBP–σA reverses the translation inhibition capacity of dsRNA in reticulocyte extracts. The effect of different additives on the translational capacity of exogenous TMV RNA was evaluated. Lane 1, no mRNA added; lane 2, TMV RNA translation; lane 3, TMV RNA translation in the presence of MBP; lane 4, TMV RNA translation in the presence of MBP–σA; lane 5, TMV RNA translation in the presence of dsRNA; lane 6, as in lane 5, but dsRNA was preincubated with MBP, then added to the lysate; lane 7, as in lane 5, but dsRNA was preincubated with MBP–σA; lane 8, as in lane 5, but dsRNA and MBP–σA were added together to the lysate without preincubation; lane 9, as in lane 5, but MBP–σA was added to the lysate 5 min later than dsRNA. Positions of protein molecular mass markers are indicated on the left.

the potential of the avian reovirus protein rσA to rescue the replication of the IFN-sensitive recombinant vaccinia virus WRE3L^{-} in HeLa cells. This mutant virus lacks the E3L gene and in contrast to the wild-type vaccinia virus, its replication in HeLa cells is restricted and sensitive to IFN (Beattie *et al.*, 1995) and late in infection the mutant virus triggers apoptosis (Rivas *et al.*, 1998). To measure the ability of rσA to reverse blockage of WRE3L^{-} in infected HeLa cells, we used a previously described transient transfection–infection assay, which analyses the capability of proteins expressed from pPR35-derived plasmids to promote WRE3L^{-} gene expression in HeLa cells (Rivas *et al.*, 1998). In this assay, promotion of WRE3L^{-} gene expression is easily monitored by measuring the activity of luciferase expressed from plasmid pPR15, which contains the luciferase reporter gene under the control of the vaccinia virus late promoter p4b (Rodriguez & Smith, 1990). The plasmid vectors used for cotransfection were the empty vaccinia virus insertional vector pPR35 and its derived vectors pPR35S2 and pPR35E3L, which contain the avian reovirus S2 gene and the vaccinia virus E3L gene, respectively, expressed from the late p4b promoter and controlled by two *lac* operator sequences. pPR35 plasmids also express the *lac* repressor from the constitutively active p7·5K vaccinia virus promoter.

HeLa cell monolayers were infected with 2·5 p.f.u. WRE3L^{-} per cell and 1 h later cells were transfected with the luciferase reporter plasmid pPR15 plus one of the three vectors mentioned above. Infections were allowed to proceed for 24 h in the presence or absence of 1·5 mM IPTG, then cell extracts were prepared and luciferase activity was determined with a luminometer (Fig. 3). As expected, the levels of luciferase activity were low in uninduced cells and in IPTG-induced cells that had been transfected with the empty plasmid pPR35. However, luciferase levels augmented considerably after IPTG induction of cells transfected with the positive control plasmid pPR35E3L or the σA-encoding plasmid pPR35S2. These results strongly suggest that both avian reovirus σA protein and vaccinia virus E3L gene products are able to rescue WRE3L^{-} gene expression in HeLa cells, probably because of their ability to bind dsRNA. Our finding that this increase is slightly higher in cells expressing σA than in those expressing E3L, together with the fact that E3L products have been shown to prevent activation of both PKR and 2-5A synthetase (Ho & Shuman, 1996; Rivas *et al.*, 1998; Romano *et al.*, 1998), suggest that σA plays a critical role in modulation of the IFN-inducible antiviral enzymes.

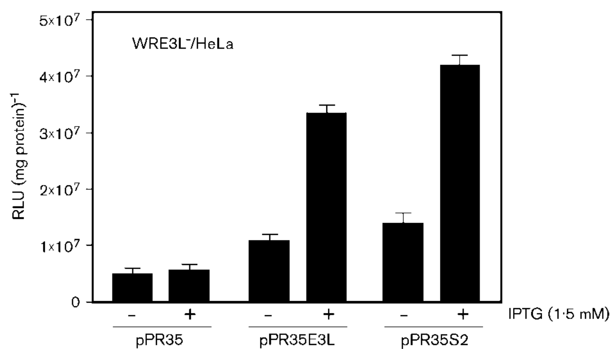

Fig. 3. Stimulation of luciferase expression. HeLa cells grown in 12-well plates were infected with 2·5 p.f.u. WRE3L^{-} per cell and 1 h later transfected with 0·2 μg per well of the plasmid pPR15 together with 2·0 μg per well of the plasmids indicated. The cells were incubated in the absence (−) or presence (+) of 1·5 mM IPTG for 24 h, then lysed and luciferase activity in the resulting extracts measured with a luminometer. The results shown are means of four independent experiments and error bars indicate standard deviations of the mean.

σA protein expressed by a recombinant vaccinia virus retains strong dsRNA-binding activity

Since vaccinia virus replication in CEFs, but not in most other cell types, is highly sensitive to IFN (Grun *et al.*, 1987; Martínez-Costas *et al.*, 2000; Youngner *et al.*, 1972), we next sought to assess whether σA protein can confer IFN resistance to vaccinia virus by comparing the IFN susceptibility of wild-type WR with that of a recombinant vaccinia virus expressing σA protein. To accomplish this, we first generated the recombinant vaccinia virus WRS2, which contains the σA-encoding gene inserted into the vaccinia virus genome under the control of the synthetic early/late PE/L promoter and then examined the capacity of this virus to express a functional rσA protein in CEFs.

A comparative electrophoretic analysis of the ^{35}S-labelled proteins synthesized in WR- and WRS2-infected CEFs (Fig. 4A) revealed that while there were no detectable differences in the protein pattern at the onset of the infection (Fig. 4A, compare lanes 1 and 2), a prominent 40 kDa radioactive protein band was detected at 8 and 24 h post-infection (p.i.) in extracts of WRS2-infected cells (Fig. 4A, lanes 4 and 6), but not in extracts of WR-infected cells (Fig. 4A, lanes 3 and 5). The 40 kDa protein comigrated with the σA protein synthesized in avian reovirus-infected cells (Fig. 4A, compare lanes 6 and 7). Immunoblot and immunoprecipitation analysis of cell extracts with polyclonal anti-σA antibodies confirmed the σA identity of the 40 kDa band present in WRS2-infected CEFs (Fig. 4B, C). Affinity chromatographic assays on poly(I:C)–agarose revealed that the rσA protein expressed by WRS2 displayed a dsRNA-binding affinity similar to that of the σA protein synthesized in avian reovirus-infected CEFs; the two proteins showed a very strong dsRNA-binding affinity, since they remained attached to the matrix after washing the dsRNA–agarose beads with a buffer containing 2 M KCl (Fig. 4D, lane 9 in panels WRS2 and S1133). Taken together, our results demonstrate that high levels of a functional σA protein are expressed by WRS2 in CEFs.

The chromatographic assay also revealed the presence in WR- and WRS2-infected cells, but not in S1133-infected cells, of low molecular weight polypeptides with high

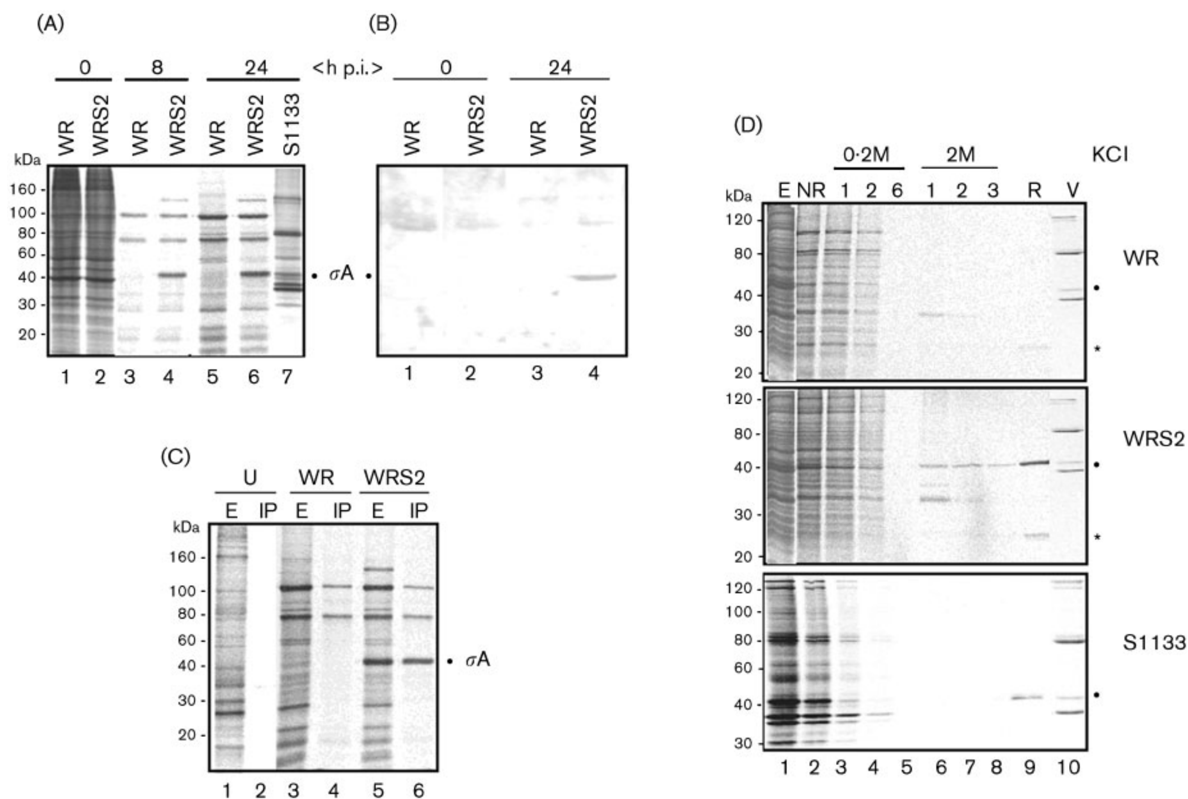

Fig. 4. Expression of WRS2 in CEFs and characterization of the rσA protein. (A) CEF monolayers were infected with 5 p.f.u. per cell of either WR (lanes 1, 3 and 5), WRS2 (lanes 2, 4 and 6) or avian reovirus S1133 (lane 7) and at the times indicated, cells were labelled for 1 h with [^{35}S]methionine and subsequently lysed. Radiolabelled proteins in cell extracts were resolved by 10 % SDS-PAGE and visualized by autoradiography. The positions of protein markers are indicated on the left and the position of σA on the right. (B) Western blot analysis of non-radioactive extracts prepared from infected CEFs at the times indicated. The position of σA protein is indicated on the left. (C) Autoradiogram of SDS-PAGE in which radiolabelled extracts of mock-infected cells (lanes 1 and 2) and infected cells (lanes 3–6) were analysed either before (lanes 1, 3 and 5) or after (lanes 2, 4 and 6) immunoprecipitation. Positions of protein markers are indicated on the left. (D) Radiolabelled extracts of CEFs infected with vaccinia virus WR and WRS2 or with avian reovirus S1133 (lane 1) were incubated with poly(I : C)–agarose beads and centrifuged. The resulting supernatants (lane 2) and the supernatants resulting from washing the beads with 0·2 M KCl (lanes 3, 4 and 5, corresponding to washes 1, 2 and 6) and with 2 M KCl (lanes 6, 7 and 8, corresponding to washes 1, 2 and 3), as well as the final pelleted beads (lane 9) and purified radiolabelled avian reovirions (lane 10) were boiled in Laemmli sample buffer and resolved by 10 % SDS-PAGE. Radiolabelled proteins were subsequently visualized by autoradiography. The positions of protein markers are indicated on the left and the positions of σA (●) and E3L gene products (*) on the right.

dsRNA-binding affinity (marked with an asterisk on the right side of the two upper panels of Fig. 4D). Based on their high dsRNA-binding affinity, their electrophoretic migration as a doublet of about 25 kDa and their absence in WRE3L$^-$-infected cells (data not shown), we believe that these protein bands correspond to the vaccinia virus E3L gene products, p20 and p25. The fact that E3L products are not able to confer vaccinia virus protection against IFN in the chicken cell suggests that their level of expression in CEFs is not sufficient to prevent activation of the IFN-inducible dsRNA-dependent enzymes. Alternatively, these products might fail to inactivate PKR because of their inability to recognize and bind the chicken PKR, since it has been demonstrated that E3L products down-regulate PKR activity partly because of complex formation (Romano *et al.*, 1998). Failure of E3L products to form complexes with avian PKR could thus be the basis for the IFN-sensitive phenotype of vaccinia virus in the chicken cell and we are currently exploring this possibility.

Protein σA protects vaccinia virus against the antiviral effects of IFN

To characterize the IFN-inhibitory activity of σA protein, we next compared the effects of rcIFN on WRS2 and WRLuc replication in CEFs. WRLuc was chosen as a negative control virus because it expresses luciferase, which lacks IFN-inhibitory activity (Gherardi *et al.*, 1999). Plaque assay

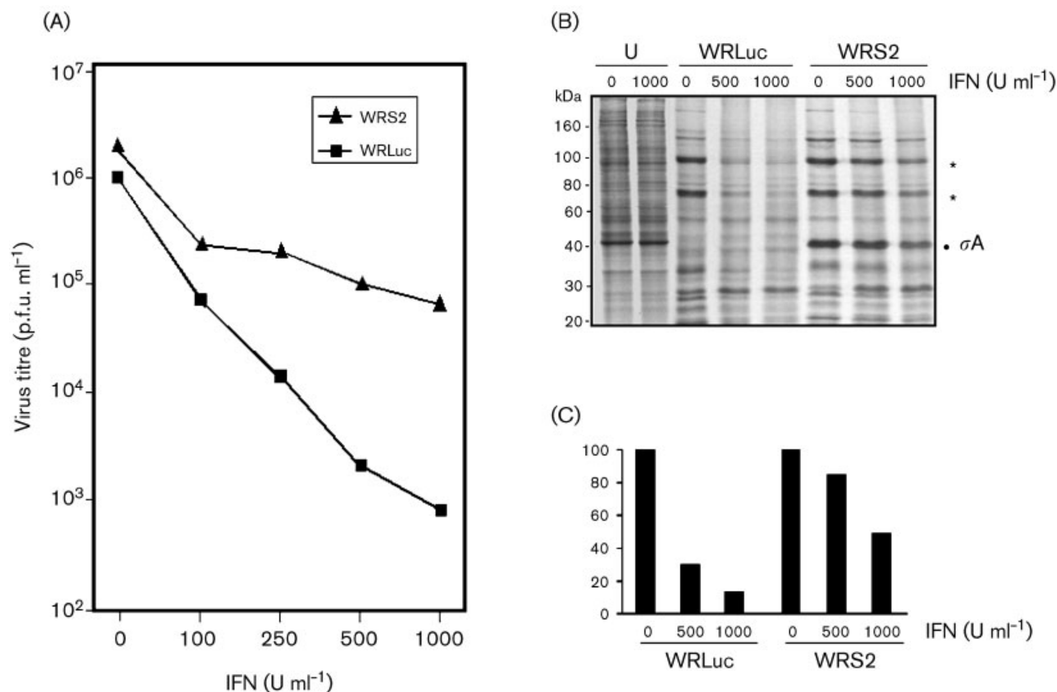

Fig. 5. Effect of rcIFN on virus replication. CEF monolayers were treated with the indicated doses of IFN and 20 h later were mock-infected (U) or infected with 0·1 p.f.u. per cell of the recombinant vaccinia viruses WRLuc or WRS2. (A) At 20 h p.i. cells were lysed and the concentration of infectious vaccinia virus particles in cell extracts was determined by plaque assay on CEF monolayers. The results shown are the means of three independent experiments. (B) At 20 h p.i. cells were labelled for 1 h with [^{35}S]methionine and lysed. The resulting extracts were analysed by SDS-PAGE and the proteins visualized by autoradiography. (C) Densitometric analysis of the two viral protein bands marked with asterisks on the right of (B). The results shown are the means of scanning autoradiograms from three separate experiments. Values are expressed as arbitrary densitometric units.

analysis of infectious progeny virus production (Fig. 5A) revealed that WRS2 replication in CEFs is much more resistant to rcIFN than WRLuc replication, suggesting that σA protein confers vaccinia virus protection against IFN. A subsequent SDS-PAGE analysis of protein synthesis in rcIFN-treated cells (Fig. 5B) showed that while the IFN treatment did not affect protein synthesis in uninfected cells (Fig. 5B, compare lanes 1 and 2) and only induced a slight reduction of viral protein synthesis in WRS2-infected cells (Fig. 5B, compare lanes 6–8), it caused a drastic inhibition of viral protein synthesis in WRLuc-infected cells (Fig. 5B, compare lanes 3–5). A densitometric analysis of the two vaccinia virus protein bands marked with asterisks on the right of the autoradiogram shown in Fig. 5(B) confirmed that viral protein synthesis in WRS2-infected CEFs was much more resistant to rcIFN than that in WRLuc-infected cells (Fig. 5C). Taken together, these results suggest that IFN inhibits replication of vaccinia virus at a translational or a pretranslational step and that expression of avian reovirus σA protein relieves such inhibition.

Since intracellular activation of the dsRNA-dependent IFN-inducible enzymes PKR and 2-5A synthetase has been shown to trigger apoptosis (Iordanov *et al.*, 2001; Castelli *et al.*, 1997; Diaz-Guerra *et al.*, 1997; Lee *et al.*, 1994), we next evaluated the capacity of rσA to interfere with activation of these enzymes by examining its capacity to prevent apoptosis induction in IFN-treated vaccinia virus-infected CEFs. The apoptotic state of these cells was assessed by examining internucleosomal DNA fragmentation into an oligonucleosomal-length DNA ladder, which is considered a reliable biochemical hallmark of apoptosis (McCarthy & Evan, 1998). The electrophoretic analysis shown in Fig. 6(A) revealed that whereas pretreatment of CEFs with IFN did not lead to DNA laddering in either uninfected (Fig. 6A, lanes 1 and 2) or WRS2-infected (Fig. 6A, lanes 6–8) cells, DNA laddering was evident in IFN-treated, WRLuc-infected cells (Fig. 6A, compare lane 3 with lanes 4 and 5). These findings suggest that the rσA protein expressed by WRS2 prevents apoptosis induction by down-regulating the activity of dsRNA-dependent enzymes.

Taken together, our findings indicate that σA protein is able to abrogate the antiviral effects of IFN, presumably through its ability to bind and sequester dsRNA from the IFN-inducible antiviral pathways, as has been reported for other dsRNA-binding proteins of viral origin. Thus, influenza virus NS1 and herpex simplex virus type 1 Us11 protein have

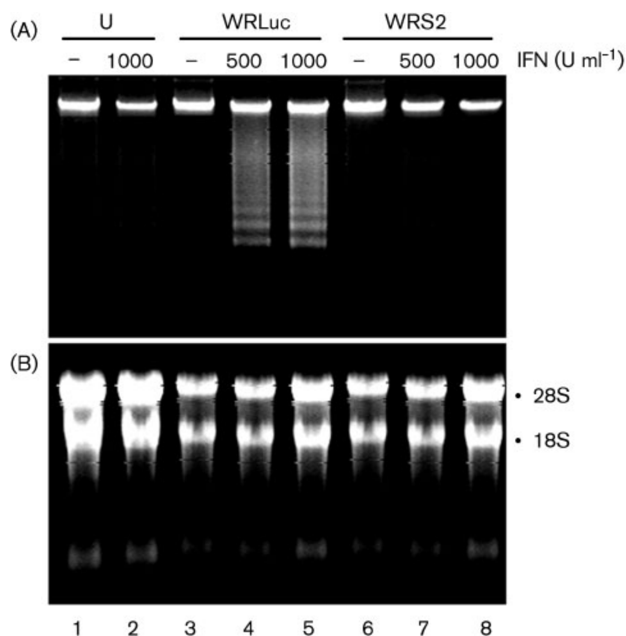

Fig. 6. Effect of rcIFN on apoptosis induction and rRNA degradation in vaccinia virus-infected CEFs. Electrophoretic analysis on agarose gels of DNA fragmentation (A) and of rRNA integrity (B), in cell extracts prepared from mock-infected CEFs (lanes 1 and 2) and from WRLuc-infected (lanes 3–5) and WRS2-infected (lanes 6–8) CEFs.

been shown to inhibit PKR activation (Hatada *et al.*, 1999; Lu *et al.*, 1995; Poppers *et al.*, 2000), whereas reovirus $\sigma3$, rotavirus NSP3 and vaccinia virus E3L have been reported to interfere with activation of both PKR and 2-5A synthetase (Beattie *et al.*, 1995; Langland *et al.*, 1994; Lloyd & Shatkin, 1992).

Since the above assays did not allow us to assess whether vaccinia virus susceptibility to IFN in CEFs is due to the activity of PKR and/or the 2-5A system, and since activation of enzymes of the 2-5A pathway has been shown to promote rRNA degradation into characteristic discrete fragments (Beattie *et al.*, 1995; Iordanov *et al.*, 2000; Rivas *et al.*, 1998; Wrescher *et al.*, 1981), we next tried to assess the possible involvement of the 2-5A/RNase L pathway by examining the integrity of 28S and 18S rRNA in vaccinia virus-infected and interferon-treated CEFs. For this, total RNA isolated from cytoplasmic extracts of mock-infected and vaccinia virus-infected cells was stained with ethidium bromide, resolved in agarose/formaldehyde gels and subsequently visualized under UV light. This experimental approach has been successfully used in our laboratories to detect degradation of rRNAs in vaccinia virus-infected cells (Esteban *et al.*, 1984; Diaz-Guerra *et al.*, 1997). As can be seen in Fig. 6(B), the IFN treatment did not promote intracellular rRNA degradation in either uninfected or WRLuc- or WRS2-infected CEFs, confirming a previously published observation that ribosomal RNA is not degraded in IFN-treated vaccinia virus-infected CEFs (Grun *et al.*, 1987). These results suggest that RNase L is not active in IFN-treated vaccinia virus-infected chicken cells and therefore that the 2-5A system is not involved in the IFN-sensitive phenotype displayed by vaccinia virus in CEFs, despite the fact that chicken IFN induces very high intracellular levels of 2-5A synthetase in these cells (Martínez-Costas *et al.*, 2000; Ball & White, 1979). This in turn would indicate both that PKR plays a key role in the IFN-sensitive phenotype displayed by vaccinia virus in CEFs and that the IFN/vaccinia virus/CEF system used in this study is a suitable system for directly testing the PKR-inhibitory activity of the product expressed by any gene inserted into the vaccinia virus genome.

Protein σA exhibits PKR-inhibitory activity

Since σA protects vaccinia virus against IFN in cells lacking RNase L activity, we next investigated whether this protection was due to interference with PKR activity. First, we evaluated the capacity of rσA to down-regulate the activity of a human recombinant PKR expressed by WR68K in BSC-40 cells. WR68K is an IPTG-inducible recombinant vaccinia virus that expresses a recombinant human PKR in BSC-40 cells (Lee & Esteban, 1993; Lee *et al.*, 1996). On IPTG induction, the expressed kinase became intracellularly activated, leading to a strong inhibition of both intracellular translation and replication of the recombinant vaccinia virus (Lee & Esteban, 1993; Fig. 7A, compare lanes 1 and 2). As expected, IPTG-induction of BSC-40 cells coinfected with WR68K and WR or with WR68K and WRLuc caused a drastic reduction in both intracellular translation (Fig. 7A, compare lanes 4 and 6 with lanes 3 and 5) and virus replication (data not shown), suggesting that PKR is not only expressed but also activated in these cells. In agreement with these findings, the intracellular levels of recombinant PKR did not increase significantly on IPTG induction (Fig. 7B, lanes 1–6), probably because the translational block imposed by the active PKR inhibits WR68K gene expression and hence synthesis of the recombinant PKR encoded by the WR68K virus. In contrast, IPTG induction of cells coinfected with WR68K and WRS2 resulted in a much less pronounced reduction of protein synthesis (Fig. 7A, compare lanes 7 and 8) and a considerable increase in the intracellular levels of recombinant PKR (Fig. 7B, compare lanes 7 and 8), indicating that PKR kinase activity is dormant in these cells and that σA protein is able to rescue vaccinia virus replication by down-regulating PKR kinase activity.

The ability of avian reovirus σA protein to inhibit PKR function was also assessed by investigating its potential to prevent apoptosis induction in WR68K-infected cells. To accomplish this, BSC-40 cells were coinfected with WR68K plus either WR, WRLuc or WRS2, then induced with IPTG; the apoptotic state of the infected cells was subsequently determined by an ELISA assay (Fig. 7C). The results of this assay revealed that apoptosis is not triggered in uninfected cells (Fig. 7C, lane 1) or in uninduced WR68K-infected cells

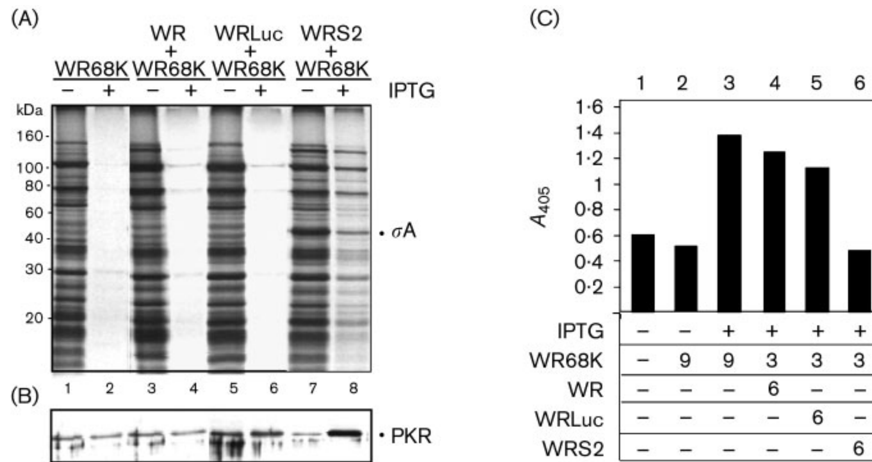

Fig. 7. PKR-inhibitory activity of rσA. (A) Translational rescue. BSC-40 monolayers were infected with 9 p.f.u. WR68K per cell (lanes 1 and 2) or coinfected with 3 p.f.u. WR68K per cell plus 6 p.f.u. per cell of the viruses indicated on top, either in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 1·5 mM IPTG. At 18 h p.i. cells were labelled for 2 h with [^{35}S]methionine and lysed. Proteins in cell extracts were resolved by 10 % SDS-PAGE and visualized by autoradiography. The positions of protein markers are indicated on the left and the position of σA protein on the right. (B) Analysis of intracellular PKR levels. The extracts shown in (A) were subjected to Western blot analysis with a polyclonal rabbit antiserum specific for human PKR. (C) Apoptotic state of infected cells. BSC-40 cells grown in 12-well plates were infected with the viruses and at the multiplicities indicated, either in the absence (lanes 1 and 2) or presence (lanes 3–6) of 1·5 mM IPTG. At 24 h p.i., cells were lysed and the extent of apoptosis was determined by an ELISA test for detection of histone-associated cytoplasmic DNA.

	1	2	3	4	5	6
IPTG	–	–	+	+	+	+
WR68K	–	9	9	3	3	3
WR	–	–	–	6	–	–
WRLuc	–	–	–	–	6	–
WRS2	–	–	–	–	–	6

(Fig. 7C, lane 2), confirming a previously published observation that vaccinia virus is not an apoptotic inducer in BSC-40 cells (Lee & Esteban, 1994). Interestingly, whereas IPTG induction triggered apoptosis in WR68K-infected cells (Fig. 7C, lane 3) and in cells coinfected with WR68K and either WR or WRLuc (Fig. 7C, lanes 4 and 5), IPTG induction did not provoke apoptosis in cells coinfected with WR68K and WRS2 (Fig. 7C, lane 6). This result further suggests that intracellular expression of σA protein down-regulates PKR activity.

Several lines of evidence suggest that σA protein down-regulates PKR function by preventing its activation, rather than by blocking its kinase activity: (i) in view of its strong dsRNA binding affinity, σA should prevent the activation of any dsRNA-dependent enzyme, as has been reported for other virus-encoded proteins (Gale & Katze, 1998); (ii) our *in vitro* translation experiments demonstrate that σA inhibits the activation, not the activity, of endogenous dsRNA-dependent enzymes in reticulocyte lysates; (iii) transient expression of σA protein rescues E3L^{-} vaccinia virus gene expression in HeLa cells, suggesting that both σA and E3L gene products use similar mechanisms for counteracting the antiviral effects of IFN; and (iv) it has recently been shown that expression of infectious bursal disease virus VP1/VP3 complexes in BSC-1 cells induces rRNA degradation because of the dsRNA polymerase activity of VP1 and that coexpression of the avian reovirus σA protein significantly reduces the rRNA degradation induced by VP1/VP3 complexes (A. Maraver, R. Clemente, J. F. Rodriguez & E. Lombardo, unpublished results) These data strongly suggest that σA is able to down-regulate PKR and the 2-5A synthetase/RNase L system *in vivo* and *in vitro* by sequestering dsRNA activators.

Like avian reoviruses, mammalian reoviruses also express a dsRNA-binding protein, the S4-encoded major outer capsid σ3 protein, and several lines of evidence have indicated that protein σ3 is likewise able to prevent PKR activation *in vivo* and *in vitro* (Bergeron *et al.*, 1998; Lloyd & Shatkin, 1992; Yue & Shatkin, 1997). However, σA possesses much stronger dsRNA-binding affinity than σ3, since the former, but not the latter, remains attached to resin-coupled dsRNA at high salt concentrations (Martínez-Costas *et al.*, 2000; Yue & Shatkin, 1997). Therefore, it would be expected that the efficiency of σA for sequestering dsRNA and preventing activation of the dsRNA-dependent enzymes is higher than that of σ3, which might account for the higher IFN sensitivity of mammalian reoviruses in comparison with avian reoviruses (Martínez-Costas *et al.*, 2000; Jacobs & Ferguson, 1991; Sherry *et al.*, 1998). This possibility is supported by the fact that the temperature-sensitive mammalian reovirus ts453 mutant, which expresses a σ3 protein with increased dsRNA-binding affinity, is more resistant to IFN than wild-type virus (Bergeron *et al.*, 1998). On the other hand, the σA equivalent in mammalian reoviruses, σ2 protein, has also been reported to be a dsRNA-binding protein, but it seems very unlikely that mammalian reovirus σ2 plays a role in IFN resistance, since although it binds dsRNA in Northwestern blotting assays

(Dermody *et al.*, 1991), it does not do so in assays performed in solution (our unpublished data). Thus, it seems that avian and mammalian reoviruses use different proteins for counteracting the antiviral action of IFN.

In conclusion, the results of the present study clearly demonstrate that σA protein possesses IFN- and PKR-inhibitory activities and further suggest that σA plays a major role in the evasion of the antiviral activity of IFN in CEFs by avian reovirus by controlling the level of dsRNA in infected cells and hence blocking cellular response pathways dependent on dsRNA. Since no consensus dsRNA-binding sequences have been found in the primary structure of σA protein and since this protein displays especially tight binding to dsRNA, studies are currently under way to map the σA dsRNA-binding domain and to assess whether the IFN-inhibitory activity of σA protein relies exclusively on its ability to sequester dsRNA.

ACKNOWLEDGEMENTS

We are grateful to Peter Staeheli for supplying rcIFN and Laboratorios Intervet (Salamanca, Spain) for providing the specific-pathogen-free embryonated eggs. We thank Aaron Shatkin and Rubén Varela for critical reading of the manuscript, Fernando Abaitua for helping us with the transient transfection assays and José Antonio Trillo for providing technical support and assistance. This work was financed by a grant from the Spanish Ministry of Ciencia y Tecnología (DGICYT, project no. PB97-0523). C. G.-L. was the recipient of a post-doctoral fellowship from the University of Santiago de Compostela.

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