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Analyzing the Mechanisms of Interferon-Induced Apoptosis Using CrmA and Hepatitis C Virus NS5A

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The dsRNA-dependent protein kinase, PKR, is a key component of interferon (IFN)-mediated anti-viral action and is frequently inhibited by many viruses following infection of the cell. Recently, we have demonstrated that IFN and PKR can sensitize cells to apoptosis predominantly through the FADD/caspase-8 pathway (S. Balachandran, P. C. Roberts, T. Kipperman, K. N. Bhalla, R. W. Compans, D. R. Archer, and G. N. Barber. (2000b) *J. Virol.* 74, 1513–1523). Given these findings, it is thus plausible that rather than specifically target IFN-inducible genes such as PKR, viruses could also subvert the mechanisms of IFN action, in part, at locations that could block the apoptotic cascade. To explore this possibility, we analyzed whether the poxvirus caspase-8 inhibitor, CrmA, was able to inhibit IFN or PKR/dsRNA-mediated apoptosis. Our findings indicated that CrmA could indeed inhibit apoptosis induced by both viral infection and dsRNA without blocking PKR activity or inhibiting IFN signaling. In contrast HCV-encoded NS5A, a putative inhibitor of PKR, did not appear to inhibit cell death mediated by a number of apoptotic stimuli, including IFN, TRAIL, and etoposide. Our data imply that viral-encoded inhibitors of apoptosis, such as CrmA, can block the innate arms of the immune response, including IFN-mediated apoptosis, and therefore potentially constitute an alternative family of inhibitors of IFN action in the cell.

INTRODUCTION

Innate immunity is a critical arm of pre-existing host defense that functions to eliminate foreign pathogens immediately after infection and subsequently directs the adaptive immune response. Key components of innate immunity, particularly against viral infection, are the interferons (IFNs), a family of pleiotropic cytokines that are divided into two major subtypes referred to as type I (IFN-α/β) and type II (IFN-γ; Stark *et al.,* 1998). Upon viral infection, the type I IFNs are transcriptionally activated and following secretion bind to their cognate receptor in either an autocrine or paracrine fashion. Dimerized IFN-α/β receptors recruit Jak1 and Tyk2 and after phosphorylation mediate the activation of STAT1 and STAT2 on phosphotyrosine residues (Darnell, 1998). The STAT 1 and STAT 2 heterodimers subsequently complex with IFN stimulating growth factor 3 gamma (ISGF-3γ/p48) forming ISGF-3, which translocates into the nucleus and binds to IFN stimulated response elements (ISREs) found in the promoters of IFN-induced genes. Through this pathway, IFN-α/β can induce >30 genes responsible for producing IFN's anti-viral and anti-proliferative effects (Stark *et al.,* 1998).

Due in part to the growing number of IFN-induced genes that are prevalent in the cell, the task of elucidating those critical genes responsible for mediating the antiviral effects of these cytokines has remained a challenge. One of the well-characterized IFN-induced proteins is the 68-kDa, dsRNA-dependent, serine/threonine kinase, PKR, a protein frequently targeted by viruses for inhibition (Meurs *et al.,* 1990). PKR undergoes autophosphorylation following interaction with dsRNA, which can comprise viral genomes or replication intermediates with substantial secondary structure. The kinase is then able to phosphorylate target substrates, the best characterized being the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which leads to the inhibition of protein synthesis in the cell (Levin, 1978). In addition to this function, PKR has been proposed to play a number of other roles in the cell, including in the regulation of selective growth factor signaling pathways and in the activation of NF-κB through modulation of IκB kinase, IKβ (Yang *et al.,* 1995; Mundschau and Faller, 1995; Kumar *et al.,* 1997; Chu *et al.,* 1999).

A consequence of activating PKR can include the induction of apoptosis or programmed cell death, a natural process required for development, homeostasis, and protection from viral infection, malignant transformation, or damaged cells. Cell death can be initiated from within

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the cell itself, as in the case of DNA or drug damage, or through the ligation of death receptors such as Fas/CD95/Apo-1, the tumor necrosis factor receptor (TNFR1), or the TRAIL receptors DR4 and DR5. For example, ligation of Fas to Fas ligand causes Fas receptor to trimerize and recruit FADD (Fas-associated death domain containing protein), which then recruits caspase-8/FLICE through its death effector domain (Chinnaiyan *et al.,* 1995; Boldin *et al.,* 1996; Muzio *et al.,* 1996). Caspase-8 is able to initiate two possible death pathways, the first of which includes the direct activation of effector caspases such as caspase-3 (Salvesen and Dixit, 1999). An alternative caspase-8 pathway can occur through the mitochondrion via activation of Bid, a proapoptotic member of the Bcl-2 family, which triggers a loss of mitochondrial transmembrane potential and potential cytochrome c release. Cytochrome c is able to form a complex with Apaf-1 and activate caspase-9 to cleave downstream executioner caspase-3, leading to cell death (Li *et al.,* 1997).

To avoid killing their host cell, viruses such as baculovirus, adenovirus, simian virus 40, and several herpes viruses have developed mechanisms to carefully orchestrate the regulation of the apoptotic cascade (Roulston *et al.,* 1999). One of the best-characterized viral inhibitors is poxvirus' CrmA, which has been shown to be a potent blocker of Fas and TNFα-induced cell death and functions by blocking caspase-8 activity (Ray *et al.,* 1992; Tewari and Dixit, 1995). Recently, our group showed that IFN and PKR appear to sensitize cells to apoptosis through the FADD/caspase-8 pathway (Balachandran *et al.,* 2000b). Thus it is plausible that viral inhibitors of the apoptotic pathway, such as CrmA, may also block an important component of IFN-mediated antiviral activity. Similarly, viral inhibitors of PKR may not only be preventing host-mediated translational repression but may also be inhibiting a mechanism of IFN-mediated cell death.

It has also recently been reported that hepatitis C virus (HCV) may inhibit PKR as a preventive measure against the antiviral effects of IFN therapy. HCV, a member of the *Flaviviridae* family, causes chronic liver disease in $\leq$85% of infected individuals, and 1.5–5.0% of those patients are estimated to develop hepatocellular carcinoma (Tanaka *et al.,* 1994; Tsukuma *et al.,* 1993). IFN or IFN combined with the nucleoside analogue ribavirin has been reported to have an optimal success rate of $\sim$40%, suggesting that HCV may directly or indirectly invoke a mechanism for subverting IFN action (Houghton, 1996). Accordingly, reports have surfaced suggesting that NS5A may play a role in repressing IFN activity (Enomoto *et al.,* 1995, 1996). In this regard, IFN-resistant strains of HCV are reported to encode a PKR binding domain, encompassing the IFN-sensitivity-determining region (ISDR; aa 2209–2248), in the NS5A viral gene product (Gale *et al.,* 1998b). Yet another possible mechanism with which HCV may subvert IFN action involves E2, an HCV glycoprotein, which has been reported to encode a PKR interacting domain that prevents the phosphorylation of eIF2α (Taylor *et al.,* 1999).

Here, we further evaluated the role of IFN and PKR in mediating cell death as well as addressed whether the viral-encoded inhibitors of host-defense, cowpox CrmA, and HCV NS5A are able to influence IFN-mediated apoptosis. To accomplish this, we used HeLa cell lines inducibly expressing NS5A from IFN-resistant and -sensitive strains or expressing CrmA to examine the effects of these viral genes on PKR activity, IFN-signaling, and IFN-mediated apoptosis. Our results demonstrate that CrmA, but not NS5A, is able to block cell death induced by either dsRNA or virus infection. The mechanism of CrmA-mediated inhibition did not involve direct inhibition of PKR or the IFN signaling pathway but functioned by inhibiting the activity of the death-inducing signaling complex (DISC), which can regulate IFN-mediated cell death. Thus viral inhibitors of FADD-dependent apoptosis may constitute an additional family of suppressors of IFN antiviral action in the cell.

RESULTS

CrmA can block IFN-induced apoptosis

Since IFN- and PKR-mediated cell death has been previously shown by our lab to be dependent on recruitment of the death adaptor molecule FADD, we examined whether CrmA, a known inhibitor of caspase-8, was capable of blocking PKR-mediated apoptosis (Balachandran *et al.,* 1998; Zhou *et al.,* 1997). To evaluate this, we established HeLa cell lines constitutively expressing CrmA in the vector pcDNA3 as well as control cell lines containing vector alone. CrmA expression was confirmed by immunoblot analysis, and each experiment was carried out using two independent cell lines to minimize the risk of obtaining a non-specific phenotype that can occur during the selection process (Fig. 1A, lanes 3 and 4). To examine whether key apoptotic pathways were intact or defective in cells expressing CrmA, or control cells carrying vector alone, cells were treated with Fas antibody, TNFα, or etoposide; this allowed us to examine death receptor and mitochondria-mediated apoptosis, respectively (Faleiro *et al.,* 1997; Li *et al.,* 1997). As has been previously described, CrmA overexpressing cells were able to block apoptosis mediated through Fas and TNFα but not by etoposide, which largely requires mitochondrial input to trigger cell death (Tewari and Dixit, 1995; Fig. 1B). These results strongly indicate that our cell lines express functional CrmA. To ensure that components of the death-induced signaling complex (DISC) were present and unaffected by transcriptional or translational constraints, expression of the pro-apoptotic proteins, Fas, FADD, and caspase-8, were measured and found to be approximately equal in each cell line examined (Medema *et al.,* 1997; Srinivasula *et al.,* 1996; Fig.

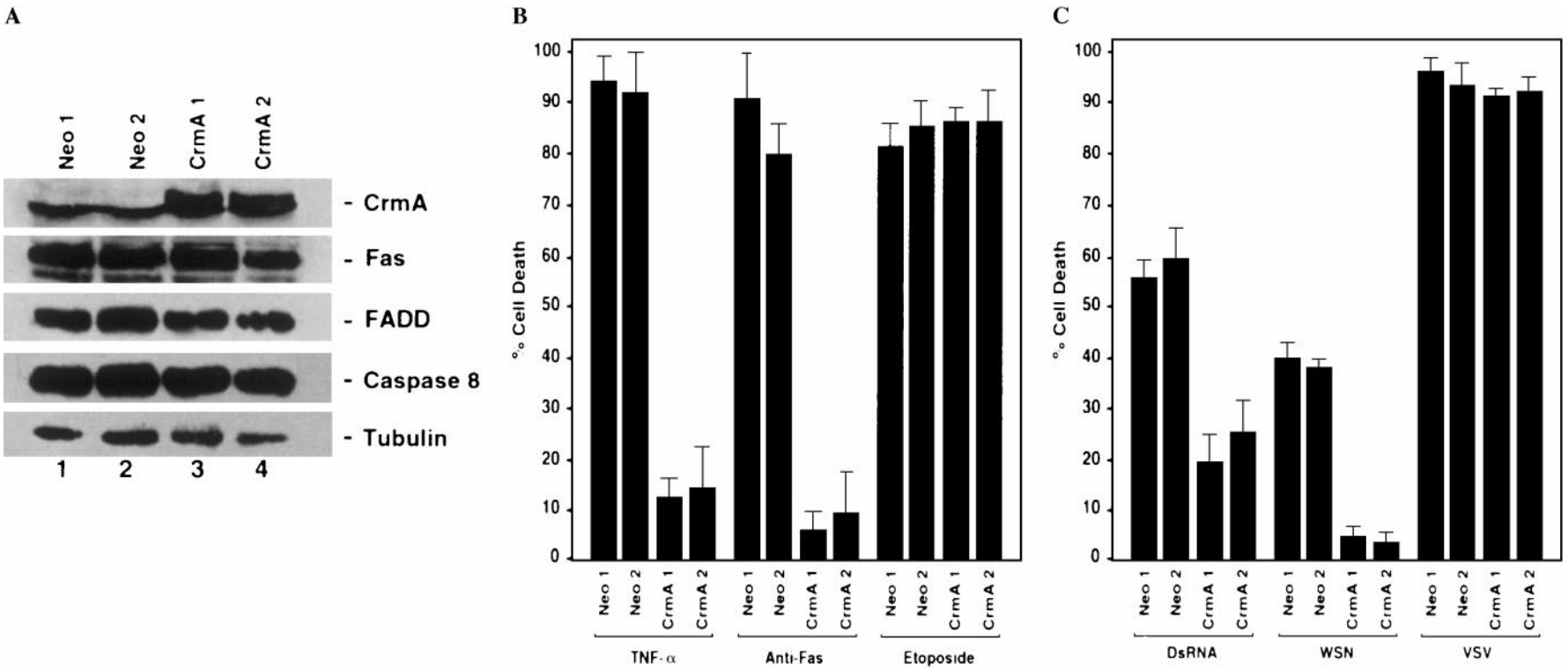

FIG. 1. (A) Expression of CrmA in clonal Hela cell lines carrying pcDNA3 vector alone (Neo 1 and 2) or vector containing poxvirus CrmA (CrmA 1 and 2) as determined by immunoblot analysis. A lower, non-specific band, cross-reacting with the available antiserum is observed in the control cells. Each of these four cell lines also expressed comparable levels of Fas, FADD, and caspase-8 protein. (B) CrmA can block TNFα- and Fas-induced cell death but not etoposide. Control and CrmA-expressing cell lines were treated with TNFα, Fas antibody, or etoposide, and cell death was measured by trypan blue exclusion. (C) CrmA blocks WSN and poly(I:C)-induced caspase-8-mediated apoptosis but not VSV. Viral- and dsRNA-induced apoptosis was analyzed by poly(I:C) transfection or infection of each cell line with VSV or WSN at a m.o.i. of 10.

1A). Our data suggest that differences in levels of apoptosis were most likely due to CrmA blocking DISC activity.

To evaluate CrmA's ability to block virus-induced cell death, each cell line was subsequently infected with vesicular stomatitis virus (VSV) or influenza A virus, strain WSN. Previous studies by our laboratory indicate that WSN and VSV appear to induce apoptosis in murine fibroblasts predominantly through the activation of DISC-associated caspase-8 or mitochondria-associated caspase-9, respectively (Balachandran *et al.,* 2000b). Accordingly, our data indicated that HeLa cells expressing CrmA blocked WSN-induced apoptosis but not VSV-mediated death, indicating that only FADD-dependent killing is predominantly affected (Fig. 1C). CrmA-expressing cells were also transfected with the synthetic dsRNA, poly(I:C), a potent activator of PKR and inducer of FADD-mediated cell death that is commonly used to mimic viral infection. As expected, CrmA-expressing cells were also able to block dsRNA-induced apoptosis, an effect strongly influenced by PKR (Balachandran *et al.,* 1998; Figs. 1C and 4). To investigate the influence of CrmA on IFN-mediated cell death, control or CrmA-expressing cells were treated with VSV or dsRNA in the presence or absence of IFN. Our data revealed that IFN pretreatment had no observable, adverse effect on control or CrmA-expressing cells in the absence of dsRNA treatment (data not shown). However, IFN-pretreated control cells subsequently transfected with dsRNA resulted in almost 100% of the cells dying within 24 h compared to ~60% in non-IFN-treated control cells (Fig. 2A). In contrast, CrmA-expressing cells exhibited greatly reduced effects of IFN/dsRNA action (approximately a 50% reduction in apoptosis compared to controls). CrmA did not affect VSV's ability to induce apoptosis, which we have shown occurs in many cell types via activation of the mitochondria-associated caspase-9 (Fig. 2A). Collectively, our data indicate that CrmA can suppress IFN-mediated apoptosis.

CrmA has no effect on PKR activity or IFN signaling

Our data indicate that CrmA is able to inhibit dsRNA-mediated cell death, an effect that is largely due to activation of PKR (Balachandran *et al.,* 1998; Der *et al.,* 1997). To investigate whether the PKR in these cell lines was functional, we analyzed the kinase's ability to autophosphorylate and phosphorylate its endogenous substrate, eIF2α. CrmA-expressing cells were labeled *in vivo* with [^{32}P]orthophosphate and transfected with poly(I:C), which potently induces the autophosphorylation of endogenous PKR (Balachandran *et al.,* 1998). PKR was subsequently precipitated using poly(I:C) agarose beads or anti-PKR polyclonal antibody. These experiments indicated that CrmA-expressing cell lines exhibited catalytically active PKR as demonstrated by the kinase's ability to autophosphorylate in response to activator (Fig. 2B, lanes 2, 4, 6). It is documented, however, that certain viral-encoded PKR inhibitors do not directly affect PKR's ability to autophosphorylate but rather prevent the kinase from phosphorylating its targets. To investigate this possibility, lysates from the above *in vivo* labeling experiment (Fig. 2B) were analyzed for eIF2α phosphorylation on Ser51 using a phospho-specific antibody to eIF2α (DeGracia *et al.,* 1997). Our data indicated that both of the control or CrmA-expressing cells exhibited phosphorylated eIF2α to varying levels after treatment with dsRNA (Fig. 2C, lanes 2, 4, 6). Total eIF2α levels were confirmed as being equal in the treated and untreated cells (Fig. 2C). These data strongly indicate that neither PKR's auto- or *trans*-phosphorylation activity is impeded by CrmA expression.

Type I IFN has been shown to potentiate apoptosis in response to dsRNA (Balachandran *et al.,* 2000b). To ensure that CrmA was not inhibiting IFN function, we examined whether CrmA had any effect on IFN signaling and thus the induction of IFN-induced genes. Accordingly, to ensure that IFN-induced gene expression was indeed normal, CrmA-expressing cells were treated with IFN-α for 4 or 8 h, and the expression of PKR, a key IFN-inducible gene was monitored. Our data indicated that PKR expression was significantly increased in each of the cell lines irrespective of whether CrmA was present or not (Fig. 2D). Densitometric analysis indicates that PKR levels rose ~2.5 fold after 8 h of IFN treatment in the CrmA-expressing cells and threefold in the controls (Fig. 2E). This translational readout indicates that the Jak/STAT IFN-signaling pathway is almost certainly functional and unimpeded by CrmA. Collectively our data infer that CrmA blocks IFN/dsRNA-mediated apoptosis downstream from IFN-gene induction and largely at the level of apoptotic, FADD-dependent signaling.

Examination of HCV NS5A in the regulation of apoptosis

Our studies indicated that CrmA is an inhibitor of dsRNA-PKR-mediated apoptosis but not of PKR itself. To complement these studies, we sought to analyze whether a reported viral-encoded PKR inhibitor, NS5A, also blocked PKR-induced apoptosis. To address this, we used HeLa cell lines that inducibly express NS5A isolated from both IFN responsive (Ies 5-genotype 1b) and non-responsive patients (1b11-genotype 1b and 1a6, 1aΔ5-genotype 1a; Polyak *et al.,* 1999, 2001). The 1aΔ5 cell line contains an ISDR deletion mutant that lacks the first 40 amino acids responsible for binding to PKR. The repression of protein expression is dependent on the presence of tetracycline or its analogue doxycycline. In the absence of doxycycline, these cell lines overexpressed NS5A, as shown in Fig. 3A.

Once we had confirmed inducible expression, we

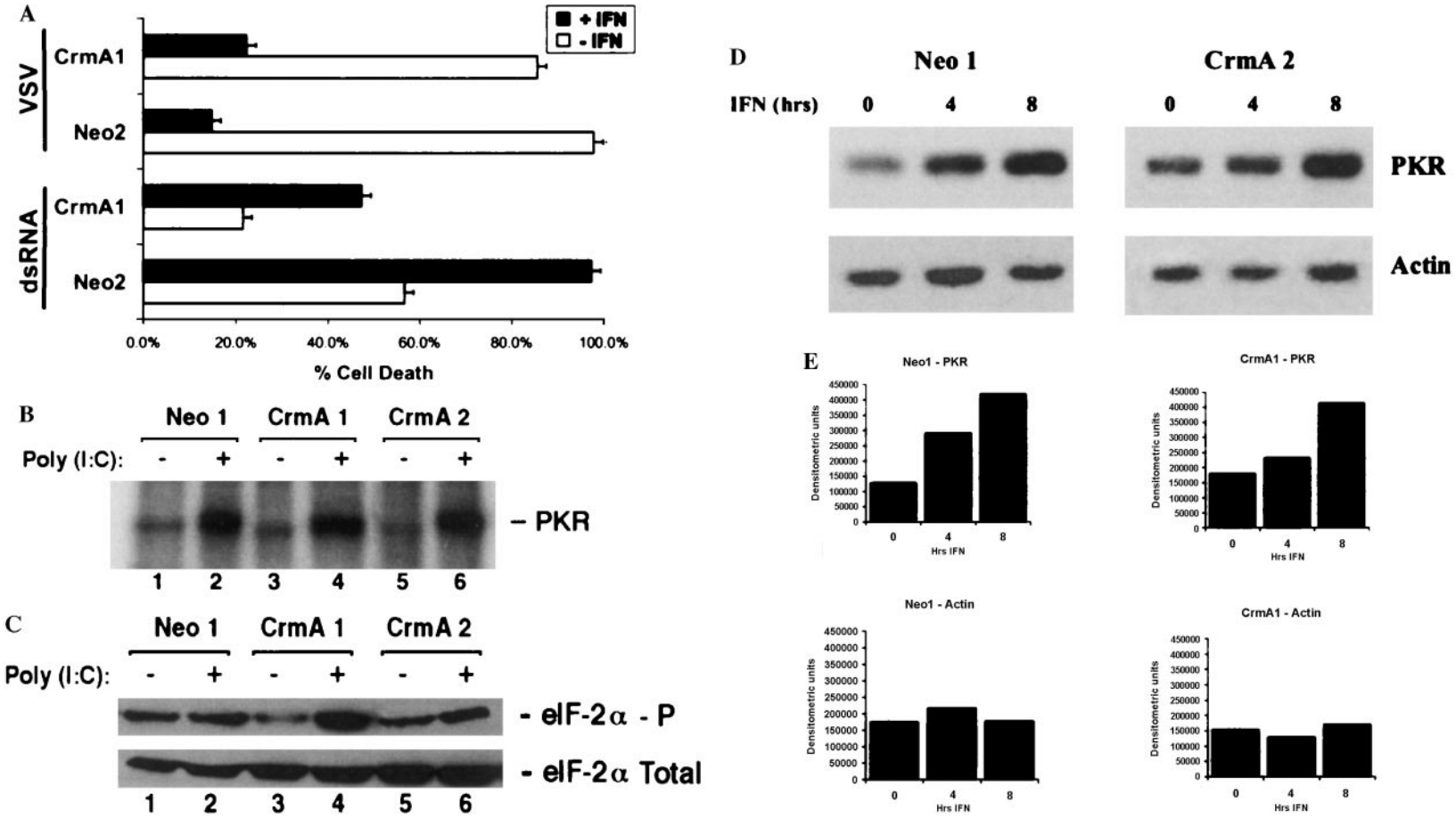

FIG. 2. (A) CrmA can inhibit IFN-mediated but not VSV-induced cell death. Control cells or CrmA-expressing cells were pre-treated with human leukocyte IFN-α (1000 unit/ml for 8 h; black columns) or left untreated (clear columns) and transfected with dsRNA or VSV. Cell death was measured by trypan blue exclusion after 15 h. (B) *In vivo* analysis of PKR and eIF2α phosphorylation after activation of PKR by dsRNA transfection. Cells were treated with dsRNA in the presence of [^{32}P]orthophosphate for 4 h. PKR was then precipitated with poly(I:C)–agarose beads. Data demonstrate that the degree of phosphorylation in the control and CrmA cells indicate that PKR is not inhibited by CrmA overexpression and is fully functional in each cell line. (C) Phosphorylation of eIF2α on Ser-51 indicates that PKR is not only capable of autophosphorylation but also *trans*-phosphorylation. (D) IFN induction of PKR, a known IFN-induced gene, is normal. Cells were treated with 1000 unit/ml of human leukocyte IFN-α for 0, 4, and 8 h. Immunoblot analysis shows a dramatic increase in expression of PKR in control and CrmA cell lines by 8 h. (E) Densitometric analysis of immunoblotted PKR and actin in IFN-treated cells as treated in (D).

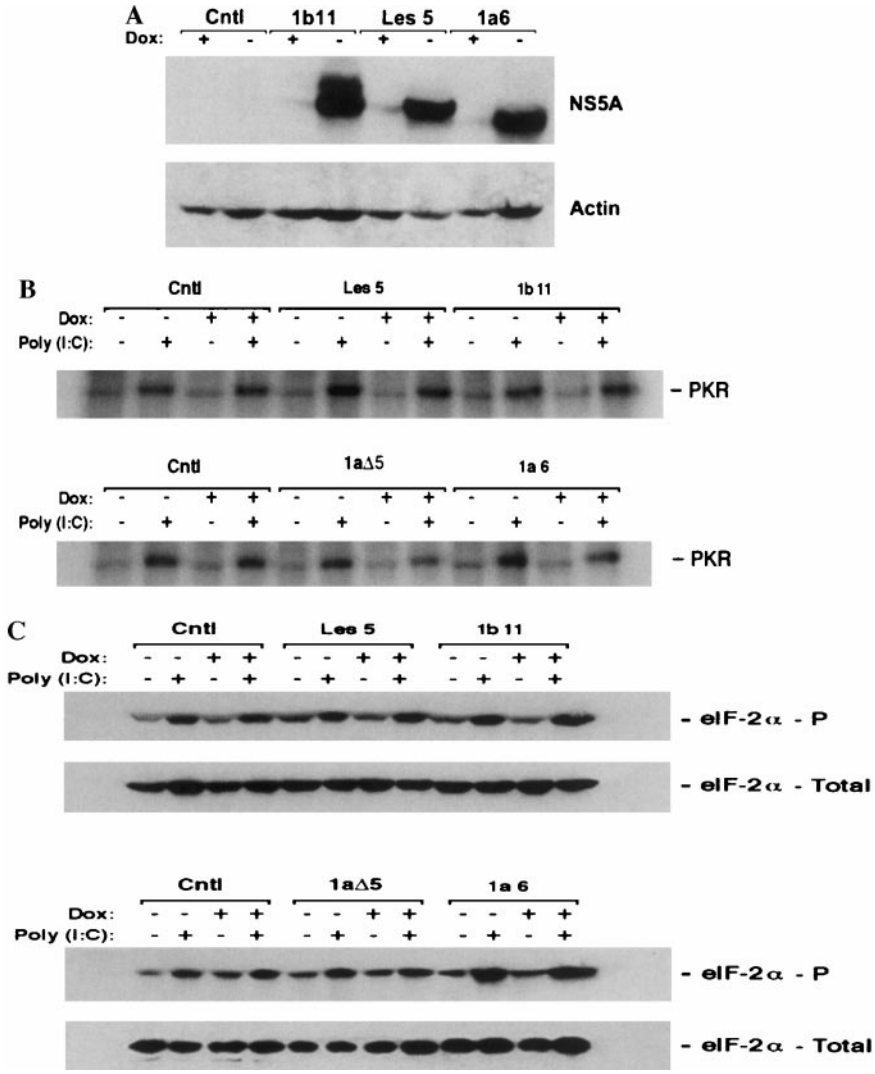

FIG. 3. (A) Inducible NS5A expression in HeLa cells. Tetracycline-inducible cells expressing NS5A were developed using Tet Off HeLa cells (Clontech, Ca). Tetracycline (or doxycycline) represses protein expression while the absence of dox allows dramatic overexpression of NS5A. Cell lines containing NS5A derived from HCV-positive patients that responded (les5) or did not respond (1b11, 1a6, and 1aΔ5) to IFN treatment (Polyak *et al.,* 1999, 2001). 1aΔ5 has a deletion mutation that removes the ISDR of NS5A, which coincides with the first 40 amino acids of the PKR binding domain. The control cells are the parental cell line for all of the inducible cells. Immunoblots were generated using human serum from HCV-positive patients. (B) PKR autophosphorylation analysis in NS5A-expressing cells. PKR was ^{32}P-labeled *in vivo* in each cell line after transfection with dsRNA. The autocatalytic activity of the appeared unperturbed in cells expressing NS5A from IFN responsive and unresponsive patients. (C) PKR phosphorylates eIF2α in NS5A expressing cells. Extracts of dsRNA-treated cells from (B) were examined for eIF2α phosphorylation by immunoblot using an antibody that recognizes phosphorylated serine 51.

evaluated NS5A's inhibition of PKR by carrying out *in vivo* labeling experiments with transfected dsRNA. Our results indicated that PKR underwent autophosphorylation whether NS5A expression from either an IFN responder (les5), non-responder (1b11 and 1a6), or missing the ISDR (1aΔ5) was repressed or not (Fig. 3B). To determine if NS5A was able to inhibit PKR at the level of substrate activation rather than autophosphorylation, we analyzed eIF2α phosphorylation in NS5A-expressing cells, in response to dsRNA. Again, we did not appreciably detect any inhibition of PKR activity in any of the NS5A-expressing cell lines (Fig. 3C). Our data would indicate that these particular cell lines, which inducibly express various representations of HCV NS5A, do not appear to contain compromised PKR/eIF2α activity in response to dsRNA treatment or to VSV infection (data not shown).

To further explore NS5A's potential role in the regulation of apoptosis, such as whether this HCV product influenced dsRNA-induced cell death, each cell line was treated with poly(I:C) in an induced and uninduced state, in the presence and absence of IFN. Principally, our data did not indicate that IFN-signaling or -induced gene expression was impaired in NS5A-expressing cells (Polyak *et al.,* submitted). In addition, HeLa cells expressing NS5A did not appear to exhibit a defect in IFN-mediated inhibition of VSV-induced apoptosis compared

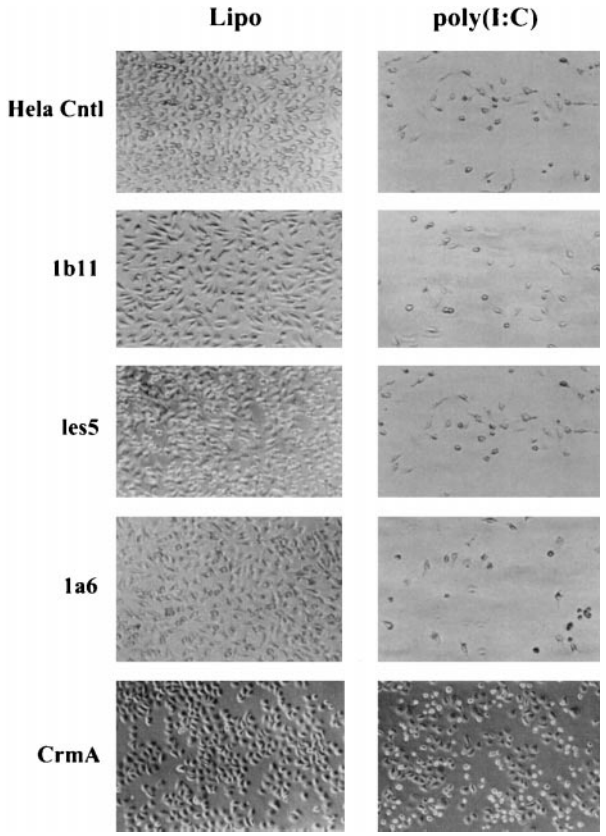

FIG. 4. Analysis of dsRNA-induced cell death in NS5A and CrmA-expressing cell lines. Each cell line was transfected with poly I:C and then examined for apoptosis 15 h post-transfection. Cell death was analyzed by trypan blue exclusion. Apoptosis of the NS5A cells was always examined in both the induced and uninduced states to rule out differences due to clonal variation. Neither NS5A from a responder (les5) or a non-responder (1b11, 1a6) patient was able to inhibit apoptosis, in contrast to CrmA.

to cells in an uninduced state (data not shown). Furthermore, as can be seen in Fig. 4, dsRNA-mediated apoptosis did not appear inhibited in cells overexpressing NS5A, and >90% of the cells died within 24 h of treatment. The effect of dsRNA was so dramatic that the addition of IFN, which enhances FADD-dependent, dsRNA-mediated cell death was masked (data not shown). Collectively, our data indicate that NS5A did not effectively prevent these cells from undergoing dsRNA-mediated apoptosis.

Finally, to further examine the apoptotic pathways of each of the inducible NS5A cell lines, cells were treated with the death ligand TRAIL and etoposide in the induced and uninduced states. Following binding to death receptors (DR) 4 and 5, TRAIL, like Fas, proceeds through the FADD/caspase-8 pathway while, as mentioned, etoposide induces DNA damage to activate mitochondria and release cytochrome c. As can be seen in Fig. 5, all of the cell lines underwent apoptosis when treated in the uninduced state, although clonal variation caused overall levels to somewhat vary. When NS5A was expressed, only slight variations were detected in TRAIL and etoposide killing, none of which correlated with IFN responsiveness (Figs. 5A and 5B).

K3L but not the ISDR of NS5A binds to PKR

NS5A has been reported to contain a stretch of 40 amino acids termed the IFN-sensitivity determining region (ISDR) that has been proposed to bind to PKR and to correlate with virus resistance to IFN therapy (Gale *et al.,* 1998a). To further investigate the potential inhibitory properties of the ISDR domain of NS5A, on PKR, we employed a coupled *in vitro* kinase assay using a model peptide substrate. A number of experiments with peptides representing PKR revealed that the peptide n-GRSRSRSRSR-c served as an effective substrate (K_m = 431 ± 85 μM; Fig. 6A). Using the coupled assay, an intrinsic ATPase activity for PKR was observed, but this was not significant compared to the consumption of ATP by peptide phosphorylation (data not shown).

We first assessed whether vaccinia virus-encoded K3L, purified as described under Materials and Methods (Fig. 6C), was able to inhibit PKR-mediated phosphorylation of the model peptide. Previous data have shown

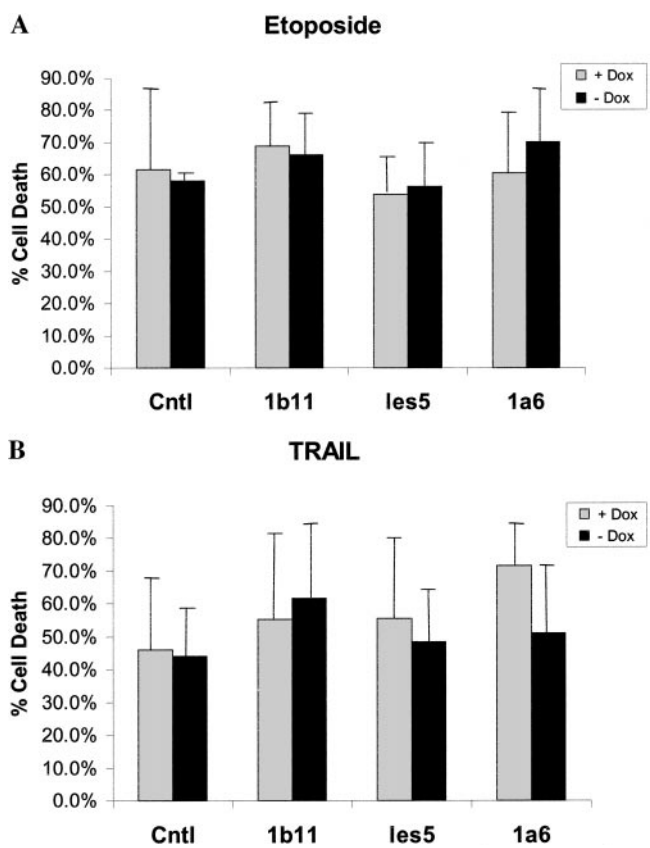

FIG. 5. NS5A HeLa cell lines are susceptible to TRAIL and etoposide-mediated apoptosis. Cells were treated in both the induced (−Dox) and uninduced (+Dox) states. Cell death analysis was conducted by trypan blue exclusion and is expressed as percentage of cells killed.

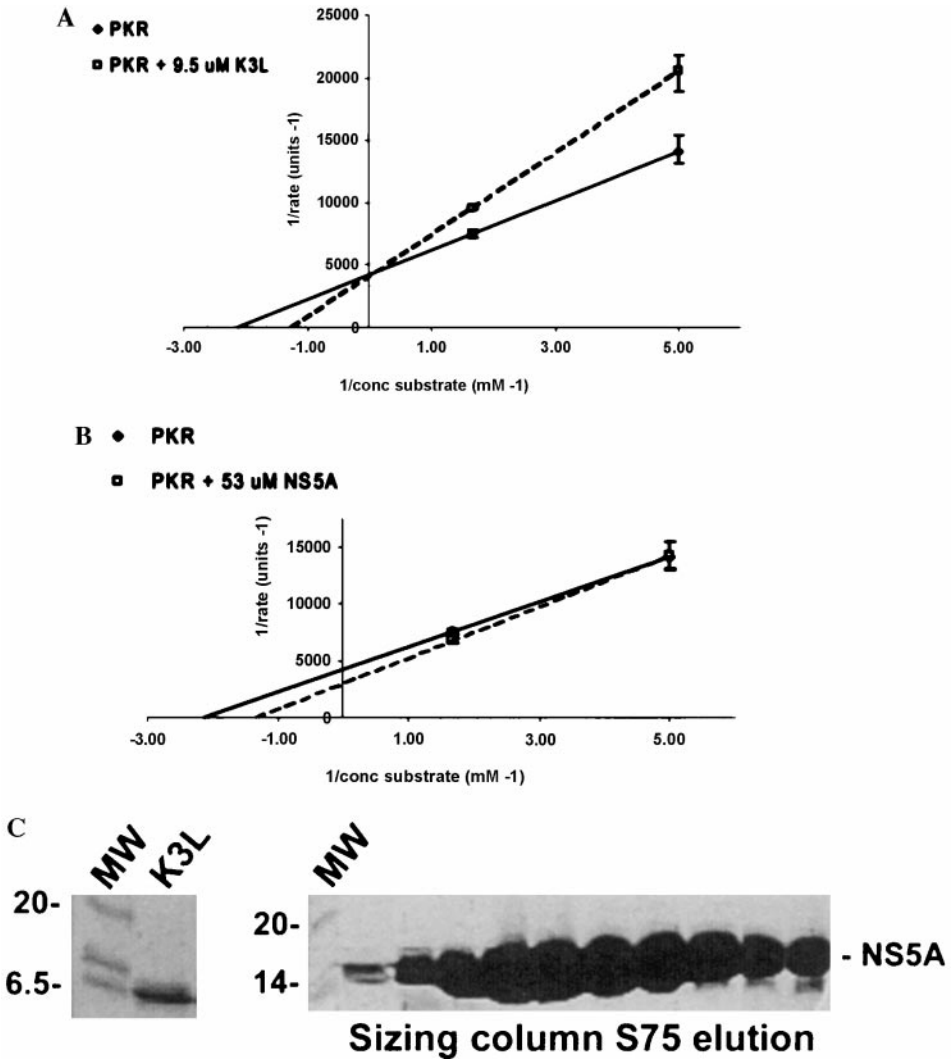

FIG. 6. Lineweaver-Burk Plots (1/[*S*] *vs* 1/[*V*]). Inhibition of PKR activity by vaccinia virus K3L but not HCV NS5A/ISDR. Lineweaver-Burk plots of the substrate concentrations and velocities are derived from the phosphorylation of RSRSRRSRSR peptide by PKR. Peptide concentrations were varied at 0.2 and 0.6 mM in the presence or absence of K3L at 9.5 μM concentration (A) or an ISDR containing NS5A protein fragment at 53 μM concentration (B). (C) Coomassie stains of purified K3L and NS5A fragment.

that K3L can inhibit PKR phosphorylation of histone IIA and eIF2α (Carroll *et al.,* 1993; Davies *et al.,* 1992). Accordingly, in Fig. 6A, we observed that at a concentration of 9.5 μM, K3L functioned as a potent competitive inhibitor of peptide phosphorylation. We next examined whether the highly purified ISDR region could inhibit the ability of PKR to phosphorylate the model peptide substrate. However, data did not indicate that this portion of the NS5A could inhibit PKR function (Fig. 6B). In fact at the highest concentration that we were able to test (53 μM), NS5A appeared to have a small stimulatory effect on PKR kinase activity. Although we cannot rule out that other portions of the NS5A product may be required to facilitate binding or that the purified ISDR may not be conformationally authentic, our data infer that amino acids 2179–2292 of HCV NS5A do not effectively inhibit PKR function *in vitro* (Gale *et al.,* 1998a).

DNA array of NS5A expression

NS5A has been reported to possess transactivation activity when fused to the Gal4 DNA binding domain (Kato *et al.,* 1997; Fukuma *et al.,* 1998; Tan *et al.,* 1999). However, its proposed function in viral replication has yet to be defined and its affects on the host cell are still being elucidated. Therefore, to further explore NS5A's role in viral pathogenesis and in regulating gene expression, we conducted a microarray analysis using the 1b11 (NS5A from a IFN treated non-responder) inducible cell line. HeLa cells expressing NS5A 1b11 were induced for 5 days, and NS5A expression was confirmed by immunoblot to ensure tight inducibility (Fig. 3A). The mRNA from uninduced cells and cells induced to express NS5A was isolated, fluorescently labeled, and hybridized to a DNA microarray containing ~10,000 genes (Alizadeh *et*

al., 2000; Iyer *et al.,* 1999). Our results, however, did not indicate that NS5A expression appreciably regulated any genes at the level of transcription. Three expressed sequence tags (ESTs) of unknown function were regulated twofold, one of which exhibited homology to the Drosophila *ariadne-2* gene, a member of a protein family that contains the Acid-rich, Ring finger, B-box, RING finger, coiled-coil (ARBRCC) motif string (Aguilera *et al.,* 2000). The remaining two upregulated genes were keratin 14, a member of the intermediate filament superfamily, and the type I inositol 1,4,5-trisphosphate receptor (IP_3R), a Ca^{2+} release channel on intracellular Ca^{2+} storage sites such as the endoplasmic reticulum (data not shown). Interestingly, no genes appeared to be downregulated by NS5A in HeLa cells greater than twofold, including a number of IFN-regulated genes such as STAT-1, PKR, and the 2′,5′ A-oligoadenylate synthetase (data not shown). However, since IFN likely induces the expression of a number of genes, it remains conceivable that as yet uncharacterized, key antiviral-induced IFN-regulated genes may be targeted by NS5A that are not represented on the DNA array. Nevertheless, our data provide a start towards further examination of the efficacy of DNA array technology in understanding the function of HCV gene products in the cell.

DISCUSSION

The IFNs constitute an intricate component of the innate immune system's ability to defend the host against viral infection (Muller *et al.,* 1994; Durbin *et al.,* 1996). Although the mechanisms of IFN-mediated antiviral activity remain to be fully clarified, our laboratory has recently shown *in vitro* that IFN-α/β is able to sensitize a number of cell types to FADD-dependent apoptosis (Balachandran *et al.,* 2000b). The investigation of IFN-mediated death could include a role for PKR since, aside from regulating the activity of eIF2α, activation of PKR, which is an IFN-induced gene, can also lead to the induction of FADD-mediated apoptosis (Balachandran *et al.,* 1998). It is not yet clear whether PKR mediates cell death through an eIF2α-dependent pathway or through an as yet unclarified mechanism involving another signaling pathway. However, activation of PKR has been reported to coincide with the stimulation of Fas expression, which presumably escapes stringent translational or post-translational constraints imposed by this kinase in the cell (Balachandran *et al.,* 1998; Donze *et al.,* 1999).

Given these findings, we postulated that viral-encoded inhibitors of apoptosis such as CrmA, a well-known inhibitor of caspase-8, may also be able to inhibit this component of IFN antiviral action, namely the induction of cell death (Quan *et al.,* 1995; Ray *et al.,* 1992). We report here that CrmA could indeed inhibit dsRNA and IFN-mediated cell death almost certainly downstream from IFN signaling and induced gene expression and at the level of DISC activity. For example, dsRNA-induced cell death, which is potently augmented by PKR, was blocked in CrmA-expressing cells even though the interferon inducibility of PKR as well as activity of this kinase was unaffected. Given these data, it is further plausible that in addition to CrmA, other viral-encoded DISC inhibitors such as the vFLIPS and E1B-19K encoded by poxviruses and herpesviruses, respectively, (Roulston *et al.,* 1999) may repress IFN-mediated apoptosis (Fig. 7).

However, it has further been reported that cells may undergo DISC-associated cell death through two potential mechanisms. For example, in cells referred to as type I, such as SKW6.4 or H9, DISC activity is strongly activated in response to apoptotic stimuli leading to direct activation of executioner caspase-3. Conversely, in type II cells, such as Jurkat and CEM, activation of the DISC is not efficient, and activation of caspase-3 proceeds predominantly through the mitochondria and can be blocked by Bcl-2 (Scaffidi *et al.,* 1998). Thus it is also possible, that in certain cells, viral-encoded inhibitors of mitochondrial-mediated cell death such as the Epstein–Barr Virus BHRF-1 or HHV-8-encoded KSBcl-2 (Henderson *et al.,* 1993; Cheng *et al.,* 1997) may also play a role in preventing IFN-induced anti-viral action (Fig. 7).

It has been previously reported that since many HCV-infected individuals do not respond to IFN therapy, HCV may have devised mechanisms to circumvent the antiviral action of these cytokines. While this escape from IFN therapy may well involve a component of the adaptive immune response, much focus has gone into elucidating a potential way in which the intracellular antiviral components of IFN action may be compromised by this virus. For example, a region within the NS5A region, termed the ISDR, has been found to correlate with IFN responsiveness in Japanese patients infected with HCV (Enomoto *et al.,* 1995, 1996). Subsequently, it has been reported that NS5A variants from HCV-infected patients not responding to standard IFN therapy may have evolved to inhibit the antiviral activity of PKR. Although our data do not support that NS5A may directly inhibit PKR, this may be a result of cell-specific or clonal differences in our cell lines that inducibly express NS5A variants as well as variations in the described assay protocols. For example, our data do not indicate significant differences in dsRNA-induced apoptosis or PKR activity whether the NS5A represents a non-responsive patient (genotypes 1a and 1b), a patient who did respond to IFN therapy (genotype 1b), or a non-responsive isolate that is lacking the ISDR (genotype 1a). Possibly the method of dsRNA-induced apoptosis, which in our hands involves liposome transportation, is very efficient, and a certain proportion of PKR may become active and overwhelm the cell to the point that NS5A is not able to interact with and inhibit every PKR molecule. Thus, low levels of activated PKR may remain available to homodimerize, which may be sufficient to induce apoptosis. Alternatively, it was re-

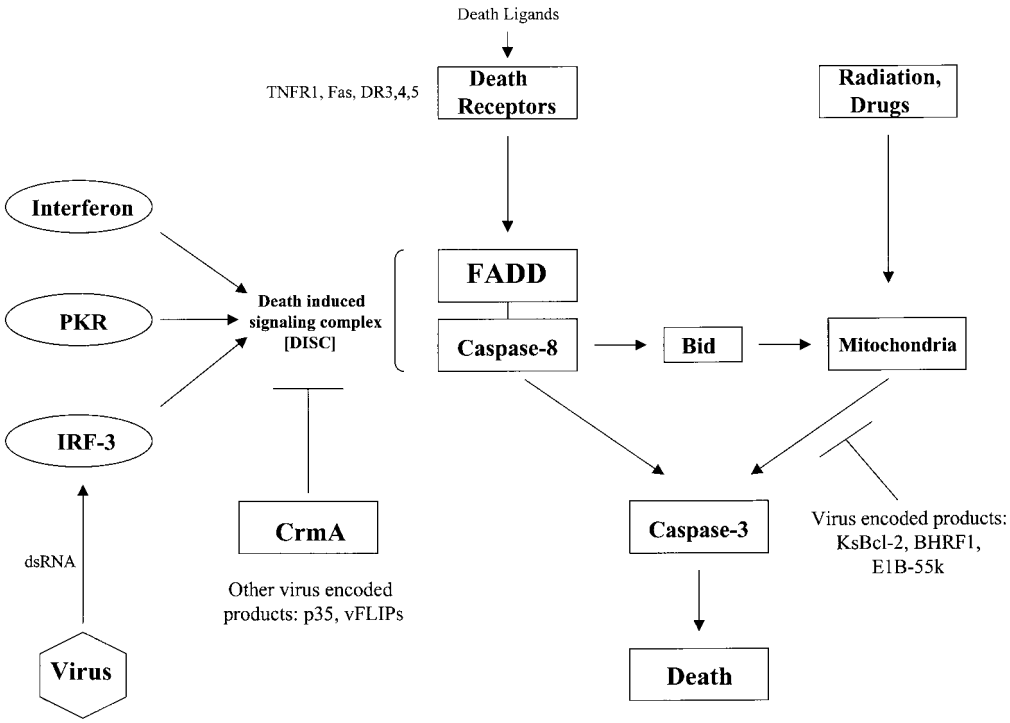

FIG. 7. Interferon and dsRNA can mediate FADD-dependent apoptosis. Viral dsRNAs can activate interferon production and induce the expression of numerous interferon-activated genes in the cell, including PKR. This event can lead to the induction of apoptosis via activation of FADD/caspase-8 (Balachandran *et al.*). Viral dsRNAs can additionally activate IRF-3, which through gene induction can also trigger apoptosis (Heylbroek *et al.,* 2000). Cowpox CrmA blocks FADD-dependent apoptosis, which can be triggered by dsRNA or death ligands such as TRAIL, by inhibiting the activity of caspase-8. Normally, caspase-8 can induce apoptosis by directly activating executioner caspase-3 as well as caspases 2, 6, and 7. Caspase-8 can also recruit mitochondrial input into the induction of cell death by activating Bid, which induces cytochrome c to release Apaf-1 modulated pro-caspase-9 to activate caspases 2, 3, 6, 7, 8, and 10 (Kroemer and Reed, 2000). Viral encoded products such as Epstein–Barr virus-encoded BHRF1 and Kaposi's sarcoma virus (HHV-8) KsBcl-2 can inhibit mitochondria-mediated apoptosis.

cently demonstrated that IRF-3, a transcriptional factor potently activated by dsRNA can also induce apoptosis in an IFN-independent manner (Heylbroeck *et al.,* 2000). Nevertheless, our data would be in agreement with the results of François *et al.,* who showed that osteosarcoma cells inducibly expressing the entire HCV genome, with corresponding proteins, do not appear to exhibit diminished PKR activity if stimulated with heparin or poly(I:C) (François *et al.,* 2000). In addition, IFN has been shown to rapidly inhibit HCV RNA replication regardless of the presence or absence of the ISDR region (Blight *et al.,* 2000). Again, it is plausible that NS5A may require cell-specific factors present only in hepatocytes to assist in any inhibitory role or even require other HCV-encoded gene products to be effective. In addition, since PKR has been proposed to play a number of roles in the cell, it remains plausible that NS5A may inhibit the IFN system by other mechanisms or prevent the interaction of PKR with alternate substrates in other signaling pathways.

In an effort to further elucidate NS5A's possible roles in pathogenesis and possibly tumorigenesis, however, we carried out a DNA array analysis on HeLa cells inducibly expressing NS5A. We observed that two genes referred to as keratin 14 and type 1 inositol 1,4,5-trisphosphate receptor (IP_3R) were moderately upregulated by NS5A expression (data not shown). It has been previously reported that cytokeratin 14 has been found in a majority of HCC cases that also express biliary markers AE1/AE3 and cytokeratin 19, suggesting that such HCCs may have de-differentiated back to their bipotential progenitor cell phenotype during transformation (Wu *et al.,* 1999). Whether keratin 14 expression is merely a characteristic of HCC or a contributor, however, has yet to be determined (Lloyd *et al.,* 1995; Sinha *et al.,* 2000; Wu *et al.,* 1999). Interestingly, a recent study has shown that induced liver fibrosis and cirrhosis can lead to an increase in isoforms 1 and 3 but not isoform 2 of the IP_3R (Dufour *et al.,* 1999). Again, whether this is a cause or result of liver injury is not yet known. Furthermore, the significance of the moderate upregulation of these genes in HeLa cells has yet to be determined. In addition to these findings, preliminary investigations did not reveal that NS5A transcriptionally repressed known molecular associates of the IFN-signaling pathway or induced genes or key members of the apoptotic pathway. Despite the fact that our data would suggest that the properties of NS5A, which have been reported to involve a role in signaling, does not affect DISC mediated apoptosis, these results do provide a start towards attempting to evaluate the role of HCV gene products in the cell.

Analysis of HCV gene expression in hepatocyte-derived cells at various time points post-expression may yield further information into the regulation of cellular gene expression by this virus.

MATERIALS AND METHODS

Cell lines

The tetracycline inducible Hela cell lines expressing NS5A 1b11, 1a6, and 1aΔ5, and NS5A les5, have been previously described (Polyak *et al.,* 1999, 2001). NS5A variants were cloned into the pTRE vector (Clontech), in which protein expression is under the control of a tTA-responsive, minimal cytomegalovirus promoter (Gossen *et al.,* 1995). Hela Tet-off cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD), 2 mM L-glutamine (Gibco), 1× antibiotic-antimycotic (100 unit/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.25 μg/ml Fungizone; Gibco), and 5 μg/ml doxycycline (Dox; Sigma, St. Louis, MO). For protein induction, cells were washed five times with phosphate-buffered saline (PBS) and incubated for 4 days in DMEM lacking Dox, supplemented with FBS. Induction was verified by immunoblot analysis using human serum from HCV-positive patients.

CrmA-expressing cell lines were generated by transfecting pcDNA3CrmA (a gift from Lawrence Boise) into Hela cells using Lipofectamine PLUS (Gibco-BRL). After a 3-h incubation, the transfection medium was replaced with DMEM and 10% FBS. The cells were allowed to recover for 24 h before transfectants were selected for with 1000 μg/ml G418 (Gibco-BRL). Colonies were isolated and expanded. CrmA expression was identified by immunoblot analysis of cell extracts using anti-CrmA antibody (Pharmingen, San Diego, CA).

Immunoblot analysis

Immunoblot analyses were performed as described by Balachandran *et al.* (1998). Protein extracts were prepared in lysis buffer I (20 mM Tris–HCl (pH 7.5), 50 mM KCl, 400 mM NaCl, 1% (v/v) N-P40, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF), 10 unit/ml aprotinin, 10 unit/ml leupeptin, and 25 mM NaF). Protein concentration was determined by Coomassie Protein Assay Reagent (Pierce Chemicals, Rockford, IL). Cell extracts were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitocellulose. Proteins were detected with primary antibodies to CrmA and β-tubulin (Pharmingen), human PKR (a gift from Ara Hovanessian), eIF2α phosphorylated and unphosphorylated (Carroll *et al.,* 1993; DeGracia *et al.,* 1997), Fas, FADD, and caspase-8 (Santa Cruz, Palo Alto, CA) and human serum from HCV-positive patients to detect NS5A (Polyak *et al.,* 1999). Secondary antibodies to mouse, rabbit, and human were conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) and visualized with chemiluminescence substrate (Pierce Chemicals).

Apoptosis and cell viability analysis

DsRNA (poly(I:C); Amersham Pharmacia Biotech, Piscataway, NJ) was prepared as previously described (Balachandran *et al.,* 2000a) and transfected using Lipofectamine PLUS (Gibco). Cells were transfected with 0.7 μg/0.5 ml DMEM, in the absence of FBS, for 3 h. Transfection medium was replaced with DMEM and 10% FBS. At 15 h post-transfection, cell viability was analyzed by trypan blue exclusion.

Fas killing was induced by treating cells with anti-human Fas monoclonal antibody (Kamiya Biomedical Company, Seattle, WA) at 2 μg/ml and protein A, also at 2 μg/ml. TNFα was used at 10 ng/ml with 2 μg/ml cyclohexamide, and etoposide treatment was 100 μM (Sigma). TRAIL-mediated killing was done using 100 ng/ml soluble TRAIL (Kamiya). Cell viability was analyzed by trypan blue exclusion at 16, 36, and 48 h, respectively.

Viral infections and titration

Vesicular stomatitis virus (VSV, Indiana strain) and influenza virus (WSN) infections and plaque assays were performed as previously described (Balachandran *et al.,* 2000b). Cells were infected with virus at a m.o.i. of 10 for cell viability assays and viral titres. Viability was assayed by trypan blue exclusion.

Analysis of PKR phosphorylation *in vivo*

Hela cells (5×10^6) were transfected with 7 μg poly(I:C) using Lipofectamine PLUS and labeled with 200 μCi/ml [^{32}P]orthophosphate (Amersham Pharmacia Biotech) for 4 h in phosphate-free DMEM (Gibco). Labeled cells were then lysed in Buffer II [25 mM Tris (pH 7.5), 25 mM KCl, 400 mM NaCl, 1% (v/v) N-P40, 1 mM EDTA, 1 mM DTT, 25 mM NaF, 200 μM PMSF, 10 unit/ml aprotinin] for 5 min, sonicated, and clarified by centrifugation. Lysates were then pre-cleared with agarose beads prior to incubation with poly(I:C)–agarose (Amersham Pharmacia Biotech) for 2 h with rotation at 4°C. The poly(I:C)–agarose beads were washed extensively with Buffer II, and bound protein was eluted by boiling in SDS protein denaturing buffer [50 mM Tris–HCl (pH 6.8), 100 mM DTT, 4% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue], resolved by SDS–10% PAGE, and visualized by autoradiography.

Bacterial expression and purification of PKR, NS5A, and K3L

The catalytic domain of PKR (residues 242–551) and an IFN-sensitivity determining region (ISDR)/PKR binding

domain containing fragment of NS5A (residues 2179–2292) were expressed in *Escherichia coli* as GST fusion proteins using derivatives of the pGEX-2T vector (Pharmacia). We have modified this vector to facilitate the cleavage of GST protein fusions using the TEV protease. Fusion constructs were transformed into BL21 bacteria and grown at 37°C to an optical density of 0.6. Protein expression was induced with 0.5 mM IPTG overnight at room temperature. The harvested cells were suspended in PBS with 0.2 mM PMSF, 3 mM MgCl, 100 Kunitz units/ml DNaseI (Roche), and lysed with an EmulsiFlex-C5 homogenizer (Avestin). Lysates were clarified by centrifugation and incubated with glutathione sepharose (Pharmacia) in batch. After extensive washing with PBS, the fusion proteins were cleaved from the sepharose matrix using TEV protease as directed by the manufacturer (Life Technologies).

Cleaved proteins were eluted and diluted threefold with buffer A (20 mM HEPES, pH 8.0) and loaded onto a 5 ml Hi-Trap S column (Pharmacia). PKR, which does not bind to the Hi-Trap S column, was concentrated and loaded onto a Sephadex 75 gel filtration column (Pharmacia) equilibrated in 20 mM HEPES, pH 7.5; 150 mM KCl; and 1 mM DTT. Elution fractions containing PKR were pooled, concentrated to 20 mg/ml, and flash frozen in liquid nitrogen for storage at −80°C. NS5A, which binds to the Hi-Trap S column, was eluted by a linear gradient against Buffer A with 1 M KCl. Fractions containing NS5A were pooled, concentrated, and loaded onto a Superdex 75 sizing column as described for PKR. Elution fractions containing NS5A protein were pooled, concentrated to 70 mg/ml, and flash frozen in liquid nitrogen. All kinetic experiments were performed with the TEV-cleaved form of PKR and NS5A. K3L was expressed and purified as previously described (Carroll *et al.,* 1993). After cleavage with TEV, the liberated NS5A and PKR proteins possessed the non-native n-terminal sequence gly, ala, his, met.

Spectrophotometric coupling assay

Kinetic analysis of bacterial expressed PKR was performed using a coupled *in vitro* kinase assay. In this assay ADP is coupled to the oxidation of NADH through pyruvate kinase (PK) and lactate dehydrogenase (LDH; Barker *et al.,* 1995). The 100-μl reaction volume contained 1 unit of LDH, 1 unit PK, 1 mM phosphoenolpyruvate, 0.2 mM NADH and 2 mM ATP in 20 mM $MgCl_2$; 0.2 mM DTT; 60 mM HEPES, pH 7.5, and 20 μg/ml BSA. Preliminary experiments ensured that the concentration of the kinase (0.008 mg/ml) was the rate-limiting component. Concurrent reactions were followed at 340 nm on a Hewlett-Packard 845-UV-Visible 7-Cell system. Protein concentration was determined by UV spectrometry at 280 nm using molar extinction coefficients calculated for each protein.

Enzyme kinetics

Kinetic constants were determined under pseudo-single substrate conditions using the general rate equation of Alberty for multi-substrate systems (Segel, 1975); $v = v\text{max}[ax][bx]/km[ax] + k\text{max} + [ax] + (k\text{sax})(km)$ Where, $v\text{max}$ = the maximal velocity when ax and b are at saturating concentration; km = the concentration that gives rise to 1/2 $v\text{max}$ when the second substrate is saturating; $k\text{sax}$ = the dissociation constant for E + AX → EAX.

At saturating concentrations of B, the general equation simplifies to a pseudo-single substrate system as follows: $V = V\text{MAX}/(1 + K\text{MAX}/[B]) = V\text{MAX}[AX]/[AX] + K\text{MAX}$ (the Michaelis Menton equation). Kinetic constants were extrapolated from Lineweaver-Burk plots of $1/[S]$ vs $1/[V]$. All reactions were repeated eight times.

DNA array of NS5A

HeLa cells expressing NS5A 1b11 were induced for 5 days in the absence of doxycycline. Approximately 8×10^6 cells were then harvested for mRNA isolation using Amersham-Pharmacia's QuickPrep mRNA Purification Kit. MRNA from cells expressing or not expressing NS5A 1b11 was then prepared according to Incyte's specifications and sent to them for microarray analysis (Alizadeh *et al.,* 2000; Iyer *et al.,* 1999).

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