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Activation of the 2-5OAS/RNase L pathway in CVB1 or HAV/18f infected FRhK-4 cells does not require induction of OAS1 or OAS2 expression

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ABSTRACT

The latent, constitutively expressed protein RNase L is activated in coxsackievirus and HAV strain 18f infected FRhK-4 cells. Endogenous oligoadenylate synthetase (OAS) from uninfected and virus infected cell extracts synthesizes active forms of the triphosphorylated 2-5A oligomer (the only known activator of RNase L) *in vitro* and endogenous 2-5A is detected in infected cell extracts. However, only the largest OAS isoform, OAS3, is readily detected throughout the time course of infection. While IFNβ treatment results in an increase in the level of all three OAS isoforms in FRhK-4 cells, IFNβ pretreatment does not affect the temporal onset or enhancement of RNase L activity nor inhibit virus replication. Our results indicate that CVB1 and HAV/18f activate the 2-5OAS/RNase L pathway in FRhK-4 cells during permissive infection through endogenous levels of OAS, but contrary to that reported for some picornaviruses, CVB1 and HAV/18f replication is insensitive to this activated antiviral pathway.

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Introduction

Hepatitis A virus (genus Hepatovirus) and coxsackieviruses (genus Enterovirus) are members of the picornavirus family of RNA viruses which display contrasting phenotypic effects following infection in permissive cells. HAV growth is cell type restricted with permissive replication usually resulting in persistent infection (Hollinger and Emerson, 2001; Ross et al., 1991). Continuous passage of cell culture adapted HAV strains results in the emergence of faster growing strains capable of inducing a cytopathic effect (cpe) during virus replication, although cpe induction is limited to only a few cell lines such as FRhK-4 and BSC1 (Anderson, 1987; Brack et al., 1998; Cromeans et al., 1987; Lemon et al., 1991; Nasser and Metcalf, 1987; Venuti et al., 1985). Coxsackieviruses are typically cytopathic in a broad host-range of cells and are generally considered a faster replicating virus than HAV, although the establishment of a persistent/latent state for coxsackievirus infection has been reported (Pallansch and Roos, 2001; Whitton, 2002; Zeichhardt and Grunert, 2000; Chehadeh et al., 2000; Conaldi et al., 1997; Feuer et al., 2004; Heim et al., 1992; Schnurr and Schmidt, 1984).

RNA virus infection can result in activation of the 2′-5′oligoadenylate synthetase (2-5OAS)/RNase L pathway, and induction of ribosomal RNA (rRNA) cleavage is a hallmark indicator of pathway activation (Cayley et al., 1982; Han et al., 2007; Han and Barton, 2002; Nilsen et al., 1982; Rice et al., 1984; Scherbik et al., 2006; Silverman et al., 1982, 1983; Wreschner et al., 1981). This pathway is traditionally considered an essential antiviral mechanism induced in cells in response to interferon (Ghosh et al., 2000; Hassel et al., 1993; Rysiecki et al., 1989; Samuel, 2001; Zhou et al., 1998). Two major components of this pathway are the interferon-inducible enzymes 2-5OAS and RNase L, whose biochemical and functional properties have been the subject of numerous reviews (Bisbal and Silverman, 2007; Hovanessian and Justesen, 2007; Samuel, 2001; Sarkar and Sen, 2004). 2-5OAS is activated by dsRNA to synthesize a series of 2′-5′ linked oligoadenylic acid multimers of the general formula pppA(2′p5′A)n (where $n \geq 2$) that, for convenience, is called 2-5A. 5′-phosphorylated trimers (or higher oligomers) of 2-5A activate the latent endoribonuclease RNase L. Once bound with 2-5A, RNase L undergoes a conformational change leading to homodimerization and enzymatic activation. Activated RNase L has been shown to degrade viral and cellular single-stranded RNA (ssRNA). Although cellular 2-5OAS and RNase L expression may be regarded as IFN-inducible, in actuality, cell lines vary with regard to their endogenous levels and the extent to which these levels increase following IFN treatment (Hovanessian and Justesen, 2007; Justesen et al., 2000).

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The 2-5OAS family of enzymes has been described for a variety of animal species including human, mouse, rabbit, rat, and pig (reviewed in Hovanessian and Justesen, 2007; Justesen et al., 2000; and Sarkar and Sen, 2004). In human cells there are three genes that encode the three major forms of the enzymes: the 40 and 46 kDa small isoforms (OAS1), the 69 and 71 kDa medium isoforms (OAS2), and the 100 kDa large isoform (OAS3). Alternative splicing of *OAS1* and *OAS2* gene transcripts yield mRNAs that encode for isoforms whose respective proteins differ only at their carboxy-terminal regions. Only one mRNA species has been identified for human *OAS3*. While human *OAS* genes appear to share certain regulatory elements such as an interferon responsive element (ISRE), they also appear to contain elements unique to particular isoforms which account for their differential response to transcription factors such as NFκB and IL-6 (Justesen et al., 2000). Indeed, the pattern of both endogenous and IFN-inducible expression of each isoform can vary with cell type suggesting the presence of additional regulatory elements that have yet to be fully elucidated. All three protein isoforms synthesize 2-5A in response to activation by dsRNA but differ in a number of aspects including the number of protein units per homo-complex, the activating concentrations of dsRNA and the range of oligomeric 2-5A units synthesized (Hovanessian and Justesen, 2007; Marie et al., 1997; Player and Torrence, 1998; Sarkar and Sen, 1998). As 2-5A is the only known activator of RNase L, its synthesis by dsRNA-activated OAS is presumed to be a necessary prerequisite for RNase L activation. Synthetic dsRNAs such as poly (I): poly (C) are useful in the study of 2-5OAS activation since they are believed to mimic OAS-activating viral dsRNA that might exist as a consequence of inherent genomic dsRNA structure (e.g., reovirus) or that may be formed as part of the replication complex of single-stranded RNA viruses, or as a consequence of secondary structures generated from viral RNA folding (Gribaudo et al., 1991; Jacobs and Langland, 1996; Marie et al., 1990; Minks et al., 1979; Nilsen et al., 1982; Rice et al., 1984). Picornavirus replication has been reported to be particularly sensitive to inhibition by an activated 2-5OAS/RNase L pathway in an interferon-dependent manner. However, these studies have been done with only a limited number of cell lines and picornaviruses (Bisbal and Silverman, 2007; Jacobs and Langland, 1996; Player and Torrence, 1998; Samuel, 2001). In contrast to these reports, we have observed the induction of rRNA cleavage, indicative of activated RNase L, during permissive infection with either CVB1 or HAV cytopathic strain (18f) in rhesus monkey kidney cell line (FRhK-4) (Goswami et al., 2004; Kulka et al., 2003). However, results from these studies also suggested that the activation of RNase L might be occurring through a non-canonical mechanism since i) there was a paucity of detectable OAS1 or OAS2 mRNA expression during HAV infection, ii) activation of the RNase L pathway (viz. rRNA degradation) did not require pretreatment of infected cells with interferon, and iii) activated RNase L had little effect on the production of infectious HAV/18f virus. The results indicated that in stark contrast to the role of RNase L in other systems, in FRhK-4 cells infected with HAV/18f, it might actually play a role in the growth and replication of this cytopathic HAV strain.

The present study was undertaken to determine whether activation of RNase L during CVB1 or HAV/18f infection in FRhK-4 cells involves activation of 2-5OAS (i.e., 2-5A synthesis) and whether activation of the 2-5OAS/RNase L pathway requires interferon. In addition, we sought to determine whether or not this pathway lacks the functionality as an antiviral mechanism against CVB1 infection as we previously reported for HAV/18f infected cells (Goswami et al., 2004; Kulka et al., 2003). Our results indicate that during either CVB1 or HAV/18f infection: i) endogenous levels of 2-5 OAS, predominantly consisting of the largest isoform OAS3, are sufficient for induction of 2-5A synthesis and activation of the 2-5OAS/RNase L pathway by virus infection; ii) interferon plays little if any role in the activation of the pathway; iii) activation of RNase L does not result in substantial viral RNA degradation (Kulka et al., 2003) or viral protein synthesis inhibition; and iv) high yields of infectious virus are obtained despite RNase L activation. We conclude that activation of the canonical 2-5OAS/RNase L antiviral pathway occurs, but does not function as part of a cellular defense mechanism against CVB1 or HAV infection in these cells.

Results

Induction of rRNA cleavage in FRhK-4 cells during CVB1 or HAV/18f infection

We have previously shown that FRhK-4 cells are permissive for CVB1 and HAV replication and that rRNA cleavage in these cells can be detected at 6–7 h pi or 24 h pi with either CVB1 or HAV/18f respectively without prior treatment with IFN (Kulka et al., 2003). The pattern of RNA cleavage was characteristic of an activated RNase L (Cayley et al., 1982; Goswami and Sharma, 1984; Goswami et al., 2004; Han et al., 2007; Han and Barton, 2002; Kulka et al., 2003; Nilsen et al., 1982; Rice et al., 1984; Scherbik et al., 2006; Silverman et al., 1982, 1983; Wreschner et al., 1981). In the current study, we used agarose gel electrophoresis to monitor rRNA cleavage as an indicator of RNase L activation and western blot analysis to monitor virus capsid expression as an indicator of virus replication in 2–8 h CVB1 infected, 16–48 h HAV/18f infected and HAV/HM 175 clone 1 persistently (100–150 d pi) infected FRhK-4 cells. Mock infected cultures served as negative controls in both rRNA degradation and western blot analyses.

In either CVB1 or HAV/18f infected cell extracts, fully processed virus capsid protein is detected and increases with the time of infection indicating successful virus infection and replication in these cells (Fig. 1, panels A and B). As previously reported, rRNA cleavage is readily observed in CVB1 infected cells at 8 h pi and in HAV/18f infected cells at 24 h pi (Kulka et al., 2003). However, in this study we observed that the appearance of rRNA cleavage during HAV/18f infection can be detected as early as 16 h pi, albeit with a lesser intensity than observed at 24 h pi (Fig. 1D). HAV/18f precursor and fully processed capsid proteins are detected at <16 h pi in the absence of rRNA cleavage (data not shown). The results indicate that the cytolytic viruses CVB1 and HAV/18f induce rRNA degradation around the time of, or prior to, the onset of cytopathic changes (cpe observed at ≥ 8 h pi for CVB1 and 36–48 h pi for HAV/18f) and virus replication–as measured by a marked increase in capsid protein expression–appears unaffected by RNase L activation in these cells (Fig. 1, panels A and B). As a positive control for induction of RNaseL-dependent rRNA cleavage, FRhK-4 cells transfected with dsRNA resulted in rRNA cleavage with a cleavage pattern similar to those observed in either CVB1 or HAV/18f infected cells (Fig. 1 panels C–E). These results suggest that in FRhK-4 cells, both viral-induced and dsRNA-induced cleavage of rRNA occur by a mechanism that is indicative of RNase L activation. The noncytolytic HAV strain HM175 establishes a persistent infection in these cells without activation of RNase L activity, despite the presence of viral capsid proteins (Fig. 1, panels F and G), and therefore, viral mRNA as well.

RNase L protein is present during CVB1 or HAV infection in FRhK-4 cells

The cleavage of rRNAs observed following infection with either CVB1 or HAV/18f indicates that the normally latent, endogenous RNase L was activated during infection with these viruses in FRhK-4 cells (Fig. 1, panels C and D). Using PCR primers that target sequences flanking the entire RNase L coding region, we confirmed by RT-PCR that an RNase L mRNA similar to that of human origin is constitutively expressed in FRhK-4 cells (Fig. 2A). Western blot analyses using RNase L specific antibody revealed readily detectable levels of RNase L protein (80 kDa) in FRhK-4 cells as well as in 293 cells (Fig. 2B). Thus, RNase L from monkey and human cells is similar with respect to the size of its mRNAs and proteins, and activation following dsRNA

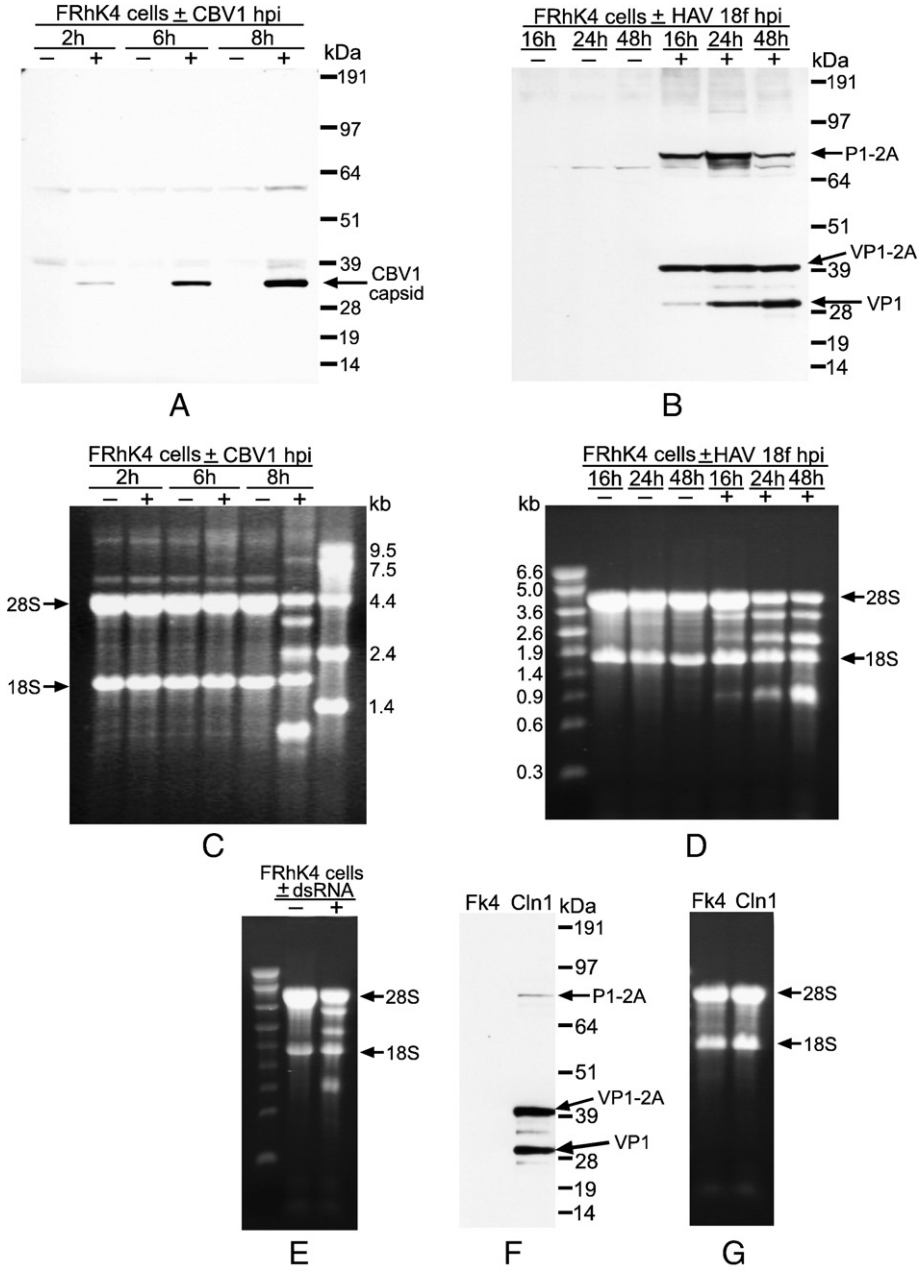

Fig. 1. Analysis of virus capsid expression and cellular rRNA cleavage in FRhK-4 cells infected with CVB1, HAV/18f or HAV clone 1. (A, B, F) Protein extracts (35 μg) were analyzed for specific virus capsid protein synthesis in mock or CVB1 infected, 2–8 h post-infection (h pi) (A), mock or HAV/18f infected, 16–48 h pi (B) FRhK-4 cells, and uninfected or HAV clone 1 persistently infected FRhK-4 cells (F), by western blot analysis using anti-CVB1 (A) or anti-HAV VP1 (B and F) antibody. Cells were infected at an moi of 8–10 for this and all experiments. Viral capsid proteins are identified with arrows. (C–E, G) Total cytoplasmic RNA (4 μg) extracts from mock or CVB1 infected, 2–8 h pi (C), mock or HAV/18f infected, 16–48 h pi (D), mock or dsRNA transfected (E), and uninfected or HAV clone 1 persistently infected (G) FRhK-4 cells were analyzed for RNA degradation by agarose gel electrophoresis (Goswami et al., 2004). The 28S and 18S rRNAs are identified with arrows. Cleavage of rRNA is not observed in mock infected, uninfected or HAV clone 1 infected cells.

treatment (data not shown). In order to confirm that RNase L is present under conditions that induce rRNA cleavage, we investigated the status of RNase L protein expression in CVB1 as well as HAV/18f infected FRhK-4 cells. As shown in Fig. 2C, CVB1 infection does not significantly alter (≤2-fold reduction by densitometric scanning) the overall level of RNase L protein compared to mock infected cells. These results indicate that RNase L is present both before and during CVB1 infection despite induction of host protein synthesis shut-off, rRNA cleavage, and a decrease in the level of some host mRNAs in these infected cells (Kulka et al., 2003).

Unlike other members of the picornavirus family such as coxsackievirus, a specific virus-mediated host (translation) shut-off function has not been identified for HAV. However, HAV/18f infection does induce rRNA cleavage as well as a reduction in the level of host mRNAs such as actin and GAPDH (Kulka et al., 2003). Despite these events, HAV/18f infection does not result in any significant reduction of RNase L mRNA levels in FRhK-4 cells (Goswami et al., 2004), and as shown in Fig. 2C, HAV/18f infection has no significant effect on the steady state levels of RNase L protein compared to mock infected cells, even at 48 h pi that is >30 h after initial detection of rRNA cleavage (see Fig. 1D). RNase L protein is also present in poly(I):poly(C) dsRNA transfected FRhK-4 cells (Fig. 2D) and at levels similar (within a 2-fold difference) to either non-transfected or transfected control cells, although RNA cleavage is quite apparent at this point (Fig. 1E). The results in Fig. 2 conclusively show that RNase L (both protein and mRNA) is present in cells following CVB1 or HAV/18f infection as well as during treatment with dsRNA, and is therefore available for activation. Interestingly, the reason for the absence of rRNA cleavage

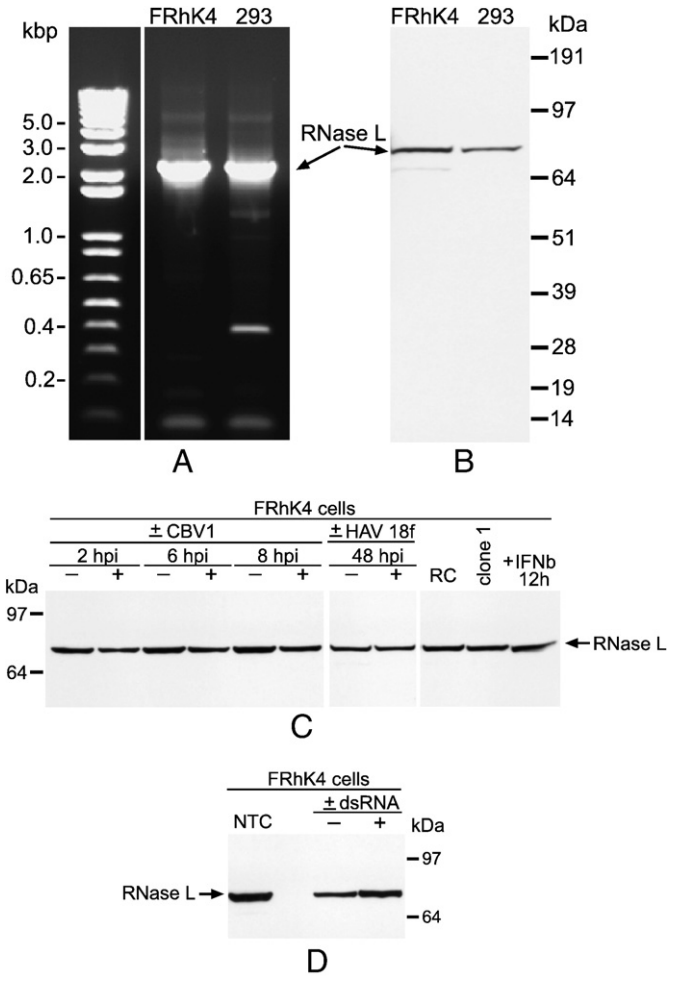

Fig. 2. RNase L expression in CVB1 or HAV infected, or dsRNA transfected FRhK-4 cells, and in uninfected human cells. (A) Total cytoplasmic RNA (1 μg) from either FRhK-4 or 293 (human) cells was subjected to reverse transcription using oligo $d(T)_{15}$ as primer for cDNA synthesis followed by PCR (30 cycles) using primers specific for RNase L followed by agarose gel electrophoresis. The positions of the RNase L amplicons are indicated by an arrow. (B–D) Protein extracts (35 μg) were analyzed for RNase L expression from routinely sub-cultured FRhK-4 or 293 cells (B); mock, CVB1, or HAV/18f infected FRhK-4 cells; routinely sub-cultured (lane RC) or HAV clone 1 persistently infected FRhK-4 cells; 12 h IFNβ (1000 U/ml) treated FRhK-4 cells (C); or 24 h non-transfected (lane NTC), mock or dsRNA transfected FRhK-4 cells (D) by western blot analysis using anti-RNase L antibody to detect the 80 kDa RNase L protein (arrow).

in persistently infected clone 1 cells is not yet known, but it is not due to an absence of RNase L or virus specific proteins as they are readily detected by western blot (Figs. 1A and 2C).

Treatment with either interferon or dsRNA, but not CVB1 or HAV/18f infection, induces expression of 2-5OAS protein in FRhK-4 cells

The transcriptional activation of cellular genes involved in an antiviral response to virus infection may be either through prior induction of interferon or through direct activation of transcription factors (Samuel, 2001; Sarkar and Sen, 2004). The 2-5OAS/RNase L activation pathway is a major antiviral pathway that may be activated by virus infection/viral dsRNA, either directly or indirectly through interferon. Regardless of whether 2-5OAS levels are induced by interferon, virus infection or dsRNA treatment, these enzymes ultimately require dsRNA for their enzymatic activation and synthesis of 2-5A oligonucleotides that go on to activate endogenous, latent RNase L (Bisbal and Silverman, 2007; Player and Torrence, 1998; Samuel, 2001). Since the induction of rRNA degradation during CVB1 or HAV/18f infection is indicative of activation of the 2-5OAS/RNase L pathway, we sought to examine whether activation might be associated with an increase in 2-5OAS expression during virus infection of FRhK-4.

In order to investigate 2-5OAS protein expression in FRhK-4 cells, we raised antiserum in rabbits against a 2-5OAS peptide whose sequence was based on the peptide B sequence derived from human 2-5OAS isoform 1 as described by Chebath et al. (1987a). As shown in Table 1, the relative position of the homologous sequences defined by the peptide is the same for both human and rhesus monkey 2-5OAS, that is, at the exon D/exon E boundary within the single (OAS1) and repeated units (OAS2 and OAS3). The percent sequence identity between the immunizing peptide and the homologous sequence in rhesus monkey OAS1 is 80%. The percent identity between the homologous regions of human OAS2 and OAS3 and the peptide immunogen ranges from 55 to 65% and 60 to 70%, respectively, and is the same between the peptide and the homologous regions of rhesus monkey OAS2 and OAS3. Since these amino acids are also highly conserved between human and rhesus monkey, we predicted that the polyclonal rabbit antiserum would recognize the epitopes in rhesus monkey 2-5OAS proteins.

Since the data in Figs. 1 and 2 suggest that dsRNA induces rRNA degradation via activation of RNase L in FRhK-4 cells, and therefore activation of the 2-5OAS, we investigated the effect of dsRNA treatment on 2-5OAS protein expression in these cells. While transfection in the absence of dsRNA treatment appears to have some negative effect on the constitutive levels of OAS3, a dramatic increase in the level of OAS3 and OAS2 proteins is observed at 24 h post-dsRNA transfection when compared to either the mock-transfected or non-transfected FRhK-4 cells (Fig. 3A). As shown in Fig. 3 (panels A–D), extracts from either untreated or mock infected FRhK-4 cells reveal that these cells contain constitutive levels of OAS3 but no detectable levels of either the OAS1 or OAS2 protein. Since we have previously reported that IFNβ treatment induces expression of OAS2 mRNA in FRhK-4 cells (Goswami et al., 2004), we included an analysis of the effect of IFNβ treatment as an additional positive control for detection of inducible OAS protein expression. FRhK-4 cells were treated for 2 h and 12 h with 1000 U/ml IFNβ prior to analysis of OAS protein expression by western blot. As observed for dsRNA transfected cells, increased expression of OAS2 and OAS3 was detected following IFN treatment (12 h) while OAS1 protein was not detected (Fig. 3B), confirming the sensitivity of these cells to IFN. These results are consistent with the interpretation that OAS expression in FRhK-4 cells

Table 1
Amino acid comparison between immunizing OAS peptide and homologous sequences in human and rhesus monkey OAS1, OAS2, and OAS3.

	OAS amino acid sequence	Percent identity	Exon boundary	GenBank accession number
OAS1	EKYLRRQLTKP/RPVILDPAD	100%	Exon D/exon E	NM016816[a]
	GKYLSRQLRKP/RPVILVPAD	80%		XM001110682[b]
OAS2	RNILLHQLQSA/RPVILDPVD	53%	Exon D1/exon E1	NM016818[a]
	RKFLLSQLQKT/RPVILDPAE	65%	Exon D2/exon E2	
	RNILLPQLQSA/RPVILDPTD	53%	Exon D1/exon E1	XM001110954[b]
	PKFLLSQLQKT/RPVILDPAE	65%	Exon D2/exon E2	
OAS3	GQFLQRQLKRP/RPVILDPAD	70%	Exon D1/exon E1	BC113746[a]
	RMHLLGQLRKP/RPLVLDPAD	60%	Exon D2/exon E2	
	GDFLKQQLQKP/RPIILDPAD	65%	Exon D3/exon E3	
	GQFLQRQLERP/RPVILDPAD	70%	Exon D1/exon E1	XM001110841[b]
	RMHLLGQLRKP/RPLVLDPAD	60%	Exon D2/exon E2	
	GDFLKQQLQKP/RPIILDPAD	65%	Exon D3/exon E3	

The OAS peptide used for immunization was (C)EKYLRRQLTKPRPVILDPAD as described under Materials and methods and based on peptide B as described in Chebath et al. (1987a). This peptide immunogen has 100% identity with human OAS1 amino acids encoded at the exon D/exon E junction. Alignment of human amino acid sequences at the exon boundaries for human (a) OAS isoforms was based on Justesen et al. (2000) while alignment of rhesus monkey (b) OAS isoforms was accomplished following sequence comparison between human and available rhesus monkey sequences. The GenBank accession numbers for OAS 1, OAS2, and OAS3 sequences are given, and amino acids identical to those in the immunizing peptide are underlined.

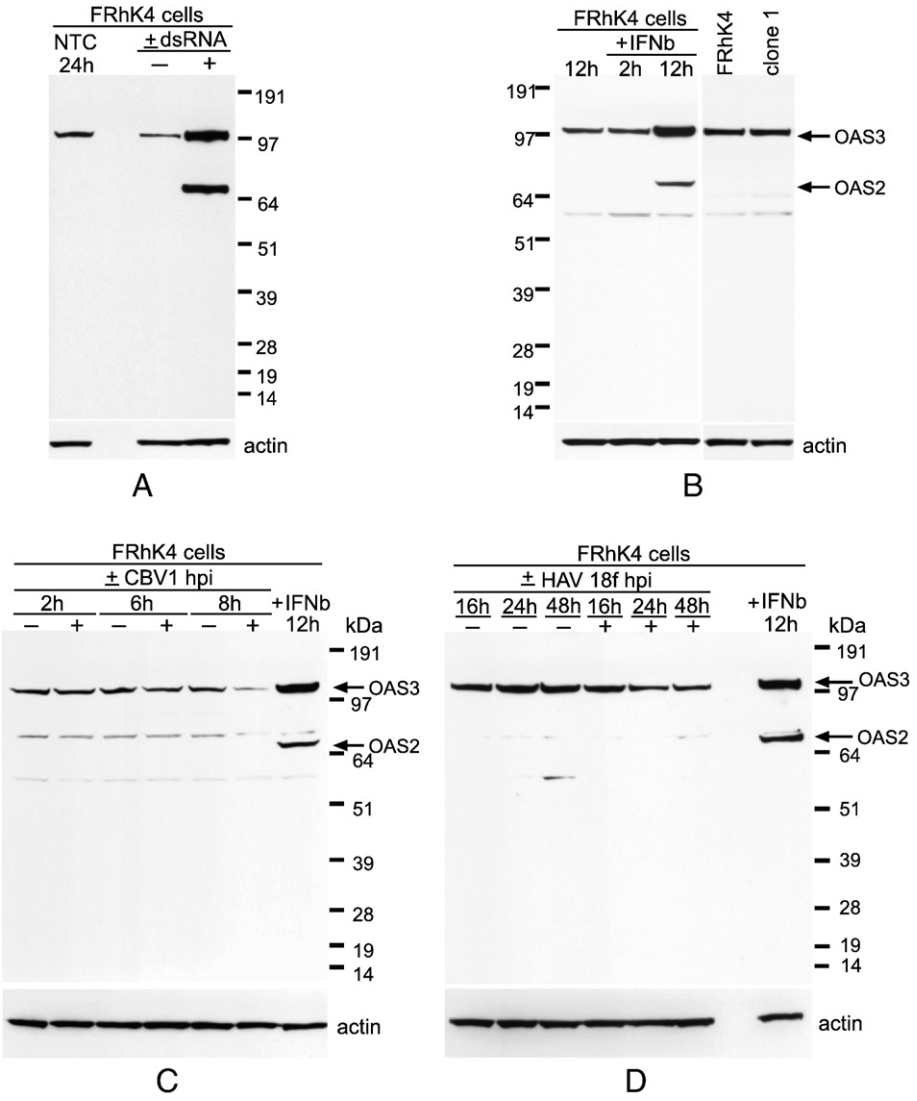

Fig. 3. The effect of CVB1 or HAV infection, IFNβ treatment, or dsRNA on OAS protein expression in FRhK-4 cells. Protein extracts (35 μg) were analyzed for OAS protein expression in 24 h non-transfected (lane NTC), mock or dsRNA 24 h-transfected (A), non-treated or IFNβ (1000 U/ml) 2 or 12 h treated (B), uninfected or HAV clone 1 persistently infected (B), mock or CVB1, 2–8 h infected (C), mock or HAV 18f, 16–48 h infected (D) FRhK-4 cells by western blot analysis using affinity purified anti-OAS antibody. The blots were stripped, washed and re-probed with anti-actin antibody (A–D, lower panels). OAS2 (69/71 kDa) and OAS3 (100 kDa) are identified with arrows, OAS1 protein expression was not detected.

following dsRNA transfection is likely through an IFN-dependent mechanism (Geiss et al., 2001; Hovanessian and Justesen, 2007; Player and Torrence, 1998; Sarkar and Sen, 2004; Samuel, 2001).

We next sought to determine whether induction of OAS expression occurs in either CVB1 or HAV infected cells. As shown in Fig. 3C, western blot analysis of protein extracts from 2 to 8 h mock infected FRhK-4 cells reveals a constitutive level of expression of the largest isoform, OAS3, which remains relatively unchanged throughout the period of mock infection and is similar to that observed in untreated FRhK-4 cells (Fig. 3, panels A and B). However, only OAS3 is detectable throughout CVB1 infection and a decrease in the level of OAS3 expression is observed at 8 h pi (Fig. 3C). All blots were stripped and re-probed with anti-actin antibody to confirm equivalent loading of samples since actin protein levels are not significantly affected by either CVB1 or HAV infection in FRhK-4 cells (Goswami et al., 2004; Kulka et al., 2003). The decrease in the level of OAS3 protein is likely due, at least in part, to host protein synthesis shut-off by CVB1 in these cells (Kulka et al., 2003).

The apparent activation of the 2-5OAS/RNase L pathway in the absence of induction of 2-5OAS protein expression in FRhK-4 cells was not unique to CVB1 since infection with a genetically distant member of the same virus *Picornaviridae* family, HAV/18f, yielded similar results. As shown in Fig. 3D, constitutive levels of OAS3 protein remain relatively unchanged throughout the period of mock infection (16–24 h) and only OAS3 is detected throughout the period of HAV/18f infection, even as late as 48 h pi when rRNA degradation is readily detected (Fig. 1D). Re-probing with anti-actin confirms equivalent loading of samples and therefore, the absence of any increase in the level OAS3 protein during HAV/18f infection. Indeed, there is some apparent decrease in the level of OAS3 observed at 24–48 h pi that may be the result of ongoing rRNA degradation. In persistently infected clone 1 cells, only the OAS3 protein is constitutively expressed and at levels similar to those detected in uninfected FRhK-4 cells (Fig. 3B). The lack of rRNA degradation in these persistently infected cells, therefore, is not due to a lack of either OAS or RNase L protein expression.

Interferon, but not CVB1 or HAV/18f, induces 2-5OAS mRNA expression in FRhK-4 cells

Since we could not exclude the possibility that either CVB1 or HAV (acute or persistent) infection may induce OAS1 or OAS2 expression at levels below the detection limit by western blot, RT-PCR was used to

examine induction of OAS expression at the level of mRNA. By applying RT-PCR using PCR primers based on the design and application by Takahashi et al. (2002) for detection of human 2-5OAS mRNA isoforms, Goswami et al. (2004) previously revealed the absence of detectable OAS1 and OAS2 mRNA in HAV infected FRhK-4 cells although the induction OAS2 mRNA levels was detected following IFNβ pretreatment. In the current investigation we focused on the application of rhesus monkey 2-5OAS specific primers for targeted amplification of each 2-5OAS isoform in order to study the differential expression of all 2-5OAS isoforms in response to CVB1 or HAV infection in FRhK-4 cells. Table 2 lists the individual primer pairs, the relative 2-5OAS mRNA nucleotide positions targeted by each primer, and the targeted exon and predicted amplicon size for each rhesus monkey primer pair.

Since western blot analysis failed to detect OAS1 and OAS2 proteins in virus infected extracts (Fig. 3, panels C and D), the PCR amplification of OAS1, OAS2, and OAS3 cDNAs was completed using 35 cycles in order to maximize detection of anticipated low basal levels of OAS1 and 2 mRNAs in these samples. RNA from control, 2 h and 12 h IFNβ (1000 U/ml) treated FRhK-4 cells was obtained for initial analysis of induction of OAS mRNA expression by RT-PCR and Fig. 4A confirms that IFNβ treatment alone does not activate the 2-5OAS/RNase L pathway in these cells despite induction in the level of OAS2 and OAS3 proteins (Fig. 3B). As shown in Fig. 4B (upper left and lower left panels), treatment of FRhK-4 cells with IFNβ (1000 U/ml) effects an increase in both OAS1 and OAS 2 mRNA above basal levels barely detected in untreated (12 h) FRhK-4 cells, with the highest levels observed at 12 h post-IFNβ treatment. The constitutive level of OAS3 mRNA appears greater than that for either OAS1 or OAS2 mRNAs (Fig. 4B) in untreated cells and no significant increase in OAS3 mRNA expression was observed following IFNβ treatment (Fig. 4B, upper right panel). Since 12 h IFNβ treatment resulted in an increase in the level of OAS3 protein, we investigated whether 35 cycles of amplification may have obscured induction of OAS3 mRNA expression by repeating the RT-PCR using 25 cycles of PCR amplification. As shown in Fig. 4B (lower right panel), induction of OAS3 mRNA is evident following 12 h IFNβ treatment when RT-PCR completed at a reduced number of PCR cycles. The results indicate that IFNβ induces expression of all three OAS mRNA isoforms in FRhK-4 cells and, with the exception of OAS1, the overall pattern and time course of OAS mRNA induction is concordant with the pattern and time course of induction of OAS protein expression (Fig. 3B versus Fig. 4B). The absence of detectable OAS1 protein following IFNβ treatment, therefore, may simply be a function of the level of sensitivity of detection by western blot analysis.

In CVB1 infected cells, there is no detectable induction of any OAS mRNA (Fig. 4C) over a 2–8 h infection period. This is not due to PCR failure since RT-PCR of RNA from 12 h IFNβ treated FRhK-4 cells, included in each analysis as a positive control, yielded readily detectable OAS1 and OAS2 amplicons. As compared to mock infected cells, OAS1 and OAS2 mRNAs remain at basal (barely visible) levels. There appears to be some decrease in the level of OAS3 mRNA in CVB1 8 h infected FRhK-4 cells, which may be due in part to the induction of RNA degradation (Fig. 1C). Nevertheless, there remains a readily detectable level of OAS3 mRNA in CVB1 infected cells. As shown in Fig. 4D, the results of RT-PCR on RNA from HAV/18f 16–24 h pi cells compared to mock infected cells gave results similar to those obtained from CVB1 infected cells whereby there is an absence of OAS1 and OAS2 mRNA induction above very low basal (to undetectable) levels and a high level of OAS3 mRNA constitutive expression that remains relatively unchanged during HAV/18f infection. The pattern of OAS3 mRNA expression parallels the profile of 2-5OAS protein expression in either CVB1 or HAV/18f infected cells. It is interesting to note that the profile of 2-5OAS mRNA expression in persistently infected HAV clone 1 cells (Fig. 4B, upper right and left, and lower left panels) is also similar to that from either CVB1 or HAV/18f infected FRhK-4 cells, supporting the conclusion that the lack of rRNA degradation in these infected cells (Fig. 1G) is not due to inhibition of either 2-5OAS protein or RNase L expression. Collectively, the data indicate that the lack of induction of OAS protein and mRNA expression in either CVB1 or HAV/18f infected FRhK-4 cells is not due to a cellular defect since both dsRNA and IFN induce expression of OAS protein and mRNA in these cells.

Interferon synthesis is not detected in CVB1 infected FRhK-4 cells

While IFN is not produced in response to either acute or persistent HAV infection in cell culture (Brack et al., 2002; Goswami et al., 2004; Kulka et al., 2003; Vallbracht et al., 1984, 1985), the production of interferon has been reported for coxsackievirus infected cell cultures and plays a role in response to infection *in vivo* (Chehadeh et al., 2000; Conaldi et al., 1997; Nakayama et al., 1989; Wessely et al., 2001). Interestingly, induction of mRNAs for all OAS isoforms occurs following IFNβ treatment (Figs. 3B and 4B), but there is an absence of OAS mRNA or protein induction during CVB1 infection in FRhK-4 cells (Figs. 3C and 4C). These results suggest a lack of interferon synthesis during virus infection in these cells. In support of this interpretation, phosphorylation of STAT-1, a hallmark cellular response to the presence of extracellular IFN (Samuel, 2001), occurs in response to treatment with either IFNα or IFNβ, but not to CVB1 infection in FRhK-4 cells (Goswami et al., 2004; Kulka et al., 2003). Furthermore, FRhK-4 cells are permissive for CVB1 replication when infected at either high (moi = 10) or low moi (moi = 0.05). This also suggests an absence of interferon production during CVB1 infection

Table 2
PCR primers for RT-PCR analysis of OAS mRNA expression in FRhK-4 cells.

OAS cDNA[a]	Primer pair[b]	Primer sequence[b]	Nucleotide position[c]	Targeted exon[d]	Amplicon size[e]
OAS1	Rmk OAS1s	5′ ACAGCTGGAAGCCTGTCAAAG 3′	+312 to +332	B	436 bp
	Rmk OAS1asb	5′ GAAATCTGTTTCCGTGCTCCC 3′	+747 to +727	D	
OAS2	Rmk OAS2s	5′ GATGAGACCATCAGGAACATCC 3′	+817 to +838	D1	484 bp
	Rmk OAS2as	5′ CCTTGATGATTCTGTACCGCTC 3′	+1300 to +1279	B2	
OAS3	Rmk OAS3sb	5′ GCCTGCTTTCTGAGTAGAGATG 3′	+2179 to +2200	E2	411 bp
	Rmk OAS3asb	5′ GGAGACTTCAAACTTGACCTCG 3′	+2589 to +2568	B3	

[a] Rhesus monkey OAS isoform cDNAs targeted by the respective primer pairs whose nucleotide sequences were obtained from GenBank; accession numbers for isoforms OAS1, OAS2, and OAS3 sequences are XM001110682, XM001110954, and XM001110841, respectively.

[b] Sense (s) and antisense (as) PCR primer pairs, and their respective nucleotide sequences, used to amplify the specific OAS isoform from FRhK-4 cells following reverse transcription using an oligo$(dT)_{15}$ primer. GCG Wisconsin programs BestFit and GAP were used to confirm identity of each primer pair against the target sequence as well as non-identity with the non-targeted isoforms of OAS.

[c] OAS mRNA nucleotide positions targeted by the sense and antisense primers where numbering starts (+1) at the first nucleotide of the initiation codon.

[d] The OAS mRNA exon containing the nucleotide sequence targeted by each primer. Exon assignment was based on the alignment of Justesen et al. (2000).

[e] The predicted size in base pairs (bp) of an amplicon generated following RT-PCR amplification of the OAS mRNA sequence with the targeting primer pair. PCR reactions contained either 2 mM MgCl (for OAS3) or 3 mM MgCl (for OAS1 and 2) with cycling parameters of 94 °C [90s], 62 °C (OAS1 primers) or 64 °C (OAS2 and OAS3 primers) [90s], 72 °C [120s] for 25 or 35 cycles; 72 °C [10 min] extension; 4 °C soak.

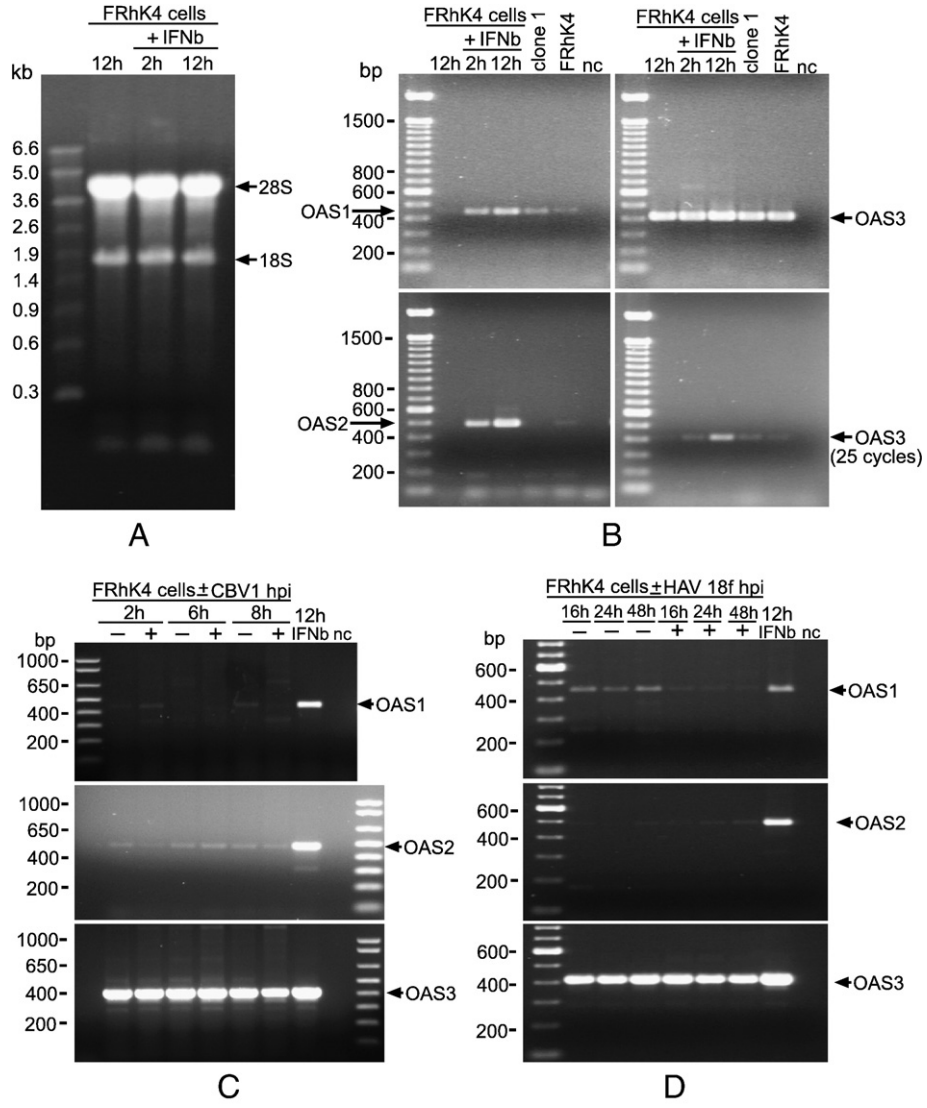

Fig. 4. The effect of CVB1 or HAV infection, or IFNβ treatment on OAS mRNA expression in FRhK-4 cells. (A) Total cytoplasmic RNA (4 μg) from untreated (12 h) or treated with 1000 U/ml human IFNβ for 2 or 12 h FRhK-4 cells was analyzed as in Fig. 1. No rRNA degradation was observed. (B–D) Total cytoplasmic RNA (1 μg) from untreated or human IFNβ (1000 U/ml) treated, 2 or 12 h (B), uninfected or HAV clone 1 infected (B), mock or CVB1 infected, 2–8 h (C), mock or HAV 18f infected, 16–48 h (D), FRhK-4 cells was subjected to reverse transcription using oligo $d(T)_{15}$ as primer for cDNA synthesis followed by PCR [35 cycles (or 25 cycles, panel B, bottom right image)] using primers specific for rhesus monkey OAS isoforms 1, 2 and 3, followed by agarose gel electrophoresis (Goswami et al., 2004). RNA from FRhK-4 cells treated for 12 h with human IFNβ (1000 U/ml) was analyzed simultaneously with RNA from mock and infected cells as a positive control, and sample-minus PCR (nc) reactions were included as negative controls. The position of OAS amplicons are indicated with arrows. The predicted amplicon sizes for rhesus monkey OAS1, OAS2, and OAS3 are 436 bp, 484 bp, and 411 bp, respectively.

since at low moi one would expect any production of IFN that occurs during the initial round of infection and replication to subsequently protect uninfected cells from subsequent infection. In contrast, FRhK-4 cells are not defective for synthesis of biologically active IFN since dsRNA treatment results in the induction of IFNβ mRNA synthesis (Goswami et al., 2004) and strong phosphorylation of STAT-1 (Fig. 5A).

We sought to directly investigate the apparent lack of interferon production by examining IFNβ mRNA expression during CVB1 infection. RNA was obtained from mock or CVB1 infected FRhK-4 cells at 2–8 h pi and analyzed by RT-PCR. Cellular RNA from dsRNA transfected FRhK-4 cells was included as a positive control for IFNβ mRNA expression since dsRNA is a well-known inducer of IFN synthesis (Sarkar and Sen, 2004). As shown in Fig. 5B, induction of IFNβ expression was not detected in CVB1 infected cells while there was a strong induction of IFNβ mRNA following dsRNA treatment. The absence of detectable IFNβ expression in mock or CVB1 infected cells was not due to a failure of the RT reaction since β-actin was readily amplified in these samples (Fig. 5B). Taken together with our previous findings (Goswami et al., 2004; Kulka et al., 2003), these results suggest that there is no significant induction/production of IFN following CVB1 infection, and therefore, IFN does not appear to play a role as an antiviral during the productive replication of CVB1 virus in FRhK-4 cells.

The lack of IFN synthesis but apparent activation of the 2-5OAS/RNase L during CVB1, or HAV/18f, infection led us to confirm whether dsRNA of viral origin is made in these cells. We used strand specific RT-PCR to detect synthesis of viral negative strand genomic RNA that is considered to be important for the formation of viral dsRNA structures necessary for the activation of the 2-5OAS/RNase L pathway and involved in induction of IFN synthesis in picornavirus infected cells (Barral et al., 2007; Gribaudo et al., 1991; Jacobs and Langland, 1996; Minks et al., 1979; Racaniello, 2001; Sarkar and Sen, 2004; Yang et al., 2007). As shown in Figs. 6A and B, both negative and positive strand RNA was readily detected in both CVB1 and HAV/18f infected cells relatively early in infection thereby providing a potential source of viral RNA for 2-5OAS/RNase L pathway activation.

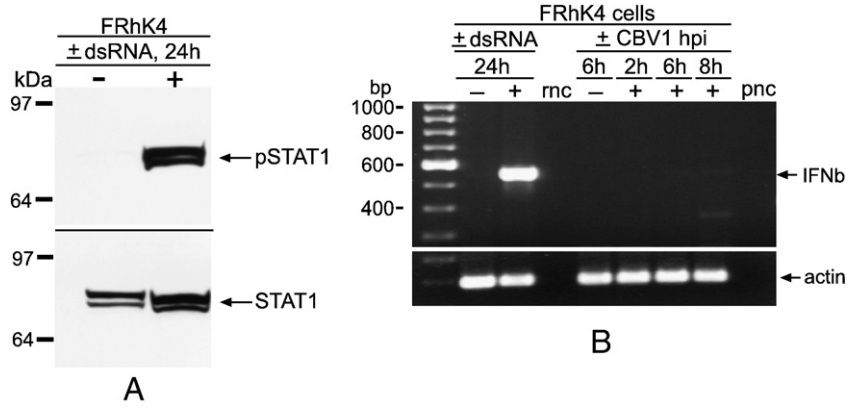

Fig. 5. The effect of dsRNA or CVB1 infection on IFNβ mRNA expression, and dsRNA on STAT-1 phosphorylation in FRhK-4 cells. (A) Nuclear (top panel) and cytoplasmic (bottom panel) protein extracts were obtained from mock (−) or dsRNA (+) transfected (24 h) FRhK-4 cells using the Nuclei EZ kit (Sigma) according to manufacturer's instructions and subjected to Western blot analysis using antibody to either phospho^{Y701}-STAT-1 (top panel) or STAT-1 (bottom panel). (B) Total cytoplasmic RNA (1 μg) from either mock or dsRNA transfected (24 h), or mock or 2–8 h CVB1 infected FRhK-4 cells was subjected to reverse transcription using oligo $d(T)_{15}$ as primer for cDNA synthesis followed by PCR using primers specific for IFNβ (top panel) or β-actin (bottom panel) and agarose gel electrophoresis as previously described (Goswami et al., 2004). The positions of the IFNβ amplicon (552 bp) and β-actin amplicon (285 bp) are indicated by arrows. RT-minus (rnc) and sample-minus (pnc) PCR reactions were included as negative controls.

Interferon treatment does not inhibit viral protein synthesis or alter induction of rRNA degradation in CVB1 infected FRhK-4 cells

The protective role of interferon in CV infection has been investigated by a number of laboratories, which have reported that coxsackievirus replication in cultured fetal heart, myocardial fibroblast and pancreatic islet cells can be inhibited by interferon (Flodstrom-Tullberg et al., 2005; Heim et al., 1992; Hultcrantz et al., 2007; Kandolf et al., 1985). We sought, therefore, to investigate whether IFN may play a protective role as an antiviral in CVB1 infected FRhK-4 cells via an interferon-mediated enhancement of rRNA degradation, inhibition of viral protein synthesis, or inhibition of virus replication following exogenous IFNβ pretreatment of virus infected cells. As shown in Fig. 7A, IFNβ pretreatment (1000 U/ml, 12 h) does not affect the temporal onset or severity of rRNA degradation during CVB1 infection (2–8 h pi) even though the same dosage and time of IFNβ treatment has been shown to elevate levels of all three isoforms of OAS (Figs. 3B and 4B). This absence of an apparent IFN effect is not due to an inhibition of CVB1 replication since the level of ^{35}S-methionine incorporation, and the pattern of labeled viral protein expression in CVB1 infected cells, were unaffected by IFNβ pretreatment as determined at 8 h pi after pulse-labeling CVB1 infected FRhK-4 cells pretreated (12 h) or not with IFNβ (Fig. 7B). We further confirmed the absence of an interferon effect on CVB1 replication by analysis of viral titers in CVB1 8 h infected (moi = 10) FRhK-4 cells pretreated or not for 12 h with 1000 U/ml IFNβ. Pretreatment with IFNβ had no significant effect (as determined by student's *t*-test, one-tailed) on virus replication since virus titers of 8.72 ± 0.12 $\log_{10}$ $TCID_{50}$/ml and 8.13 ± 0.24 $\log_{10}$ $TCID_{50}$/ml (average of two experiments, duplicate samples per time point ± 1 standard deviation) were achieved in infected cells without or with IFNβ pretreatment, respectively. In addition, we observed that IFNβ pretreatment had no effect on the time of onset (7–8 h pi) or severity of cpe (nearly all cells floating at 14–16 h pi) between CVB1 infected cells (moi = 10) pretreated (12 h) or not with IFNβ (1000 U/ml). Our findings suggest, therefore, that IFNβ does not play a significant protective role as an antiviral compound against CVB1 in these cells even though it functions to elevate components of the 2-5OAS/RNase L antiviral pathway.

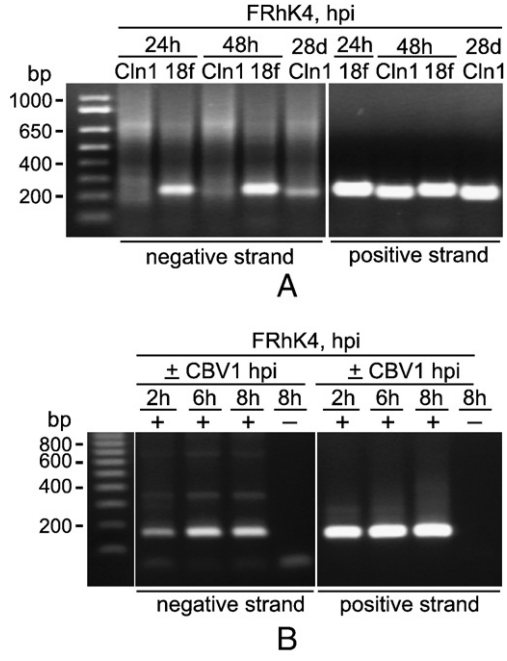

Fig. 6. Detection of viral negative strand RNA synthesis by strand-selective RT-PCR. (A) Cytoplasmic RNA was isolated from HAV/18f or clone 1 (Cln 1) infected FRhK-4 cells after acute infection (24 or 48 h) or persistent infection (28d). Reverse transcription was carried out using either the sense or the antisense primer to prime cDNA synthesis, followed by PCR amplification as described in Materials and methods. The presence of negative strand viral RNA (an indicator of RNA replication) is detected only in acute infection with the 18f virus and persistent infection by clone 1 virus (28d). The presence of viral positive strand RNA following acute infection with clone 1 virus (48 h) indicates the presence of input viral genome. (B) Cytoplasmic RNA was isolated from mock or CBV1 infected (2–8 h) FRhK-4 cells for reverse transcription with sense or antisense primers followed by PCR as described in Materials and methods. The presence of both positive and negative strand synthesis is detected throughout the time period of CVB1 infection.

Taken *in toto*, the data in Figs. 1–7 suggest that existing levels of 2-5OAS during CVB1 or HAV/18f infection are sufficient for activation by these viruses, and any increase in 2-5OAS levels prior to infection (e.g., following interferon treatment) does not enhance 2-5OAS activity, or the downstream events of OAS activation, i.e. RNase L activation and RNA cleavage.

Endogenous 2-5 OAS is functionally active in CVB1 and HAV/18f infected FRhK-4 cells

Since our results indicate that activation of the 2-5OAS/RNase L pathway occurs in virus infected FRhK-4 cells containing constitutive levels of 2-5OAS, we sought to determine whether endogenous

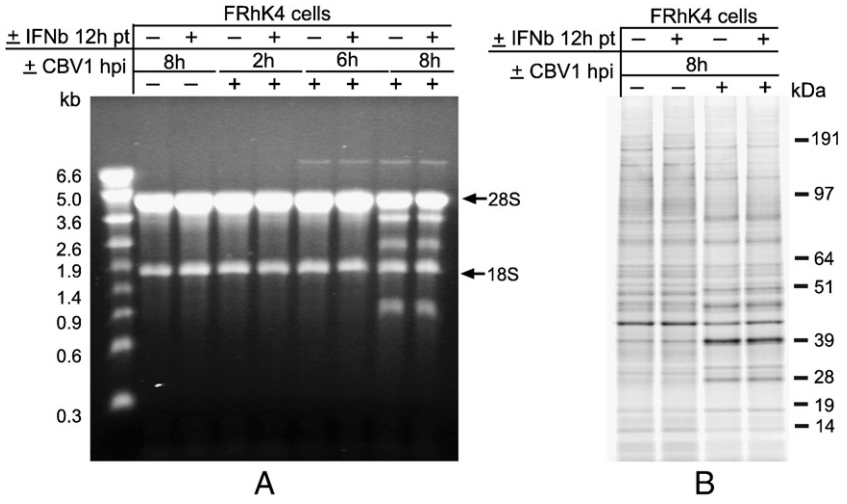

Fig. 7. The effect of IFNβ pretreatment on rRNA degradation and viral protein synthesis in CVB1 infected FRhK-4 cells. (A) Total cytoplasmic RNA extracts (4 μg) from CVB1 infected FRhK-4 cells at 2, 6 and 8 h pi, treated or not 12 h with 1000 U/ml human IFNβ prior to infection were analyzed for RNA degradation as in Fig. 1. The 28S and 18S rRNAs are identified with arrows. Pretreatment with IFNβ has no effect on the induction of rRNA degradation in FRhK-4 cells following CVB1 infection. (B) FRhK-4 cells were grown in 25 cm^2 flasks and 12 h prior to virus infection cells were treated or not with growth media containing 1000 U/ml human interferon β (Sigma-Aldrich). Cells were mock or CVB1 infected and labeled for 2 h beginning at 6 h pi with 50 μCi/ml of ^{35}S-methionine (GE Life Sciences) in methionine-free medium (EMEM, Sigma) supplemented with 5% dialyzed FBS (Sigma) following a 30 min pre-incubation in methionine-free medium. Labeled protein extracts (200,000 cpm per lane) were subjected to SDS-PAGE, the gel was fixed and treated with Amplify for signal enhancement according to manufacturer's instructions (Amersham Pharmacia Biotech Inc., Piscataway, NJ), then soaked in water containing 20% ethanol and 10% glycerol for 30 min prior to overnight drying between cellophane sheets (Diversified Biotech, Boston, MA). The dried gel was exposed to a phosphorimager screen prior to analysis using the Typhoon scanner (Molecular Dynamics Inc., Sunnyvale, CA).

2-5OAS is capable of producing authentic 2-5A. Cell extracts were obtained from CVB1 or HAV/18f infected, or interferon treated FRhK-4 cells, and analyzed for their capability of synthesizing 2-5A *in vitro* based on previously published methods that employ a synthetic dsRNA matrix, poly(I):poly(C)-agarose, for affinity capture and subsequent activation of OAS (Revel et al., 1981; Sarkar and Sen, 1998). Analysis of product formation was accomplished by denaturing polyacrylamide gel electrophoresis (Miele et al., 1991). Rabbit reticulocyte (nuclease treated) lysate was used as a positive source of endogenous OAS and a control for *in vitro* synthesis of 2-5A (Hovanessian and Kerr, 1978; Suhadolnik et al., 1983; Wu et al., 1985). As shown in Fig. 8, panel B (lanes RL), the synthesis of 2-5A from reticulocyte lysates is quite robust and results in the formation of the 2-5 A oligomer series ppp5′A (2′p5′A)n for $n \geq 2$, whereby $n = 2$ and $n = 3$, for example, represent the trimer and tetramer of 2-5A, respectively. We also examined whether we could detect synthetase activity *in vitro* in extracts derived from either CVB1 (8 h) or HAV/18f (48 h) infected, or IFNβ treated FRhK-4 cells. As shown in Fig. 8 panel B, the synthesis of 2-5A is detected in both virus infected (lanes CVB1 and 18f) and IFNβ (lane IFN) treated cell extracts. Treatment of the post-reaction mixture with RNase 1 did not alter the pattern of product formation (Fig. 8, panel A) indicating that activated 2-5OAS from CVB1 or HAV/18f infected, or IFN-treated, FRhK-4 cells synthesize authentic 2-5A oligomers that contain a 2′ to 5′ linkage resistant to RNase cleavage. The

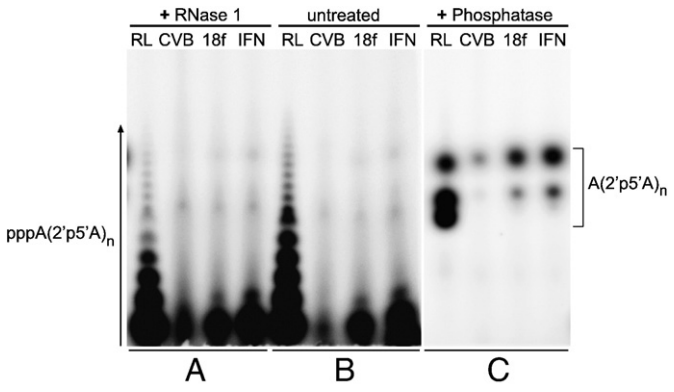

Fig. 8. Analysis of 2-5A following *in vitro* activation of endogenous OAS. (A) Rabbit reticulocyte lysate (RL) or cell lysates prepared from either CVB1 (8 h pi), HAV/18f (48 h pi) infected, or IFNβ treated cells were reacted with poly(I):poly(C)-agarose for activation of endogenous OAS in the presence of ATP (mixture of unlabeled and ^{32}P-labeled) for subsequent synthesis of 2-5A. Radiolabeled 2-5A products were analyzed by 7 M urea/20% PAGE (Miele et al., 1991) following treatment with either RNase 1 (A) or phosphatase (C), or without further treatment (B). The wet gel was exposed to a phosphorimager screen and scanned using the Typhoon scanner (Molecular Dynamics Inc., Sunnyvale, CA). The 2-5A oligomer products are identified.

Table 3
Detection of 2-5A in CVB1 and HAV/18f infected cell extracts.

Lysate[a]		2-5A pmol/dL[d]
Extract (sample)[b]	Dilution[c]	
CVB1		
10 h pi (1)	Neat	35
10 h pi (2)	Neat	31
(2)	1:2	33
HAV/18f		
24 h pi (1)	Neat	33
24 h pi (2)	Neat	63
(2)	1:2	62
48 h pi	Neat	202
72 h pi	Neat	137
Clone 1 (1)	Neat	0
(2)	Neat	0
ds RNA		
24 h (1)	Neat	>640
(1)	1:2	762
24 h (2)	Neat	>640
(2)	1:2	>730
(2)	1:8	1548

[a] Extracts from 150 cm^2 cells were prepared as lyophilized lysates (see Materials and methods) and resuspended in 0 pmol/dL 2-5A standard for duplicate analysis (50 μl per sample analysis) by RIA, yielding a final cell monolayer equivalent of 0.9 cm^2/μl per analysis.

[b] Indicates whether the first (1) or second (2) of 2 independent samples was analyzed.

[c] Resuspended lysates were analyzed undiluted (neat) or following further dilution (vol:vol) with 0 pmol/dL 2-5A standard.

[d] The concentration of 2-5A determined after background (control lysate) subtraction and correction for dilution.

electrophoretic pattern of ^{32}P-labeled 2-5A oligomers in Fig. 8 (panels A and B) also suggests that these are likely 5′ triphosphorylated species of 2-5A (Marie et al., 1997; Miele et al., 1991; Sarkar and Sen, 1998; Rebouillat et al., 1999). To confirm that these species are indeed 5′ phosphorylated, post-reaction mixtures were incubated with alkaline phosphatase prior to gel electrophoresis. As shown in Fig. 8C, the mobility of labeled 2-5A synthesized in reticulocyte lysates as well as in infected, or IFN-treated, FRhK-4 cell extracts was significantly altered and the discrimination of individual oligomer species was impaired in a manner consistent with phosphatase mediated cleavage of 5′ terminal phosphates present on 2-5A (Marie et al., 1997). In summary, the results in Fig. 8 clearly indicate that endogenous levels of 2-5OAS in either IFN-treated or virus infected FRhK-4 cells are capable of synthesizing authentic 2-5A in response to activation by dsRNA.

The activator of RNase L, 2-5A, is detected in CVB1 and HAV/18 f infected cell extracts

Ultimately, the detection of endogenous 2-5A in CVB1 or HAV/18f infected FRhK-4 cells would provide confirmation that rRNA degradation in these cells involves activation of the 2′-5′ OAS/RNase L pathway in a 2-5A dependent fashion. Therefore, detection of intracellular 2-5A was undertaken by RIA (Sawai et al., 1985) using a commercially available RIA kit with a simple modification to permit direct detection of 2-5A in heat-denatured clarified lysates obtained from virus infected cells (see Materials and methods).

As a positive control for 2-5A detection, dsRNA treated FRhK-4 cell lysates were analyzed for 2-5A levels by RIA since this compound is a known inducer of 2-5OAS-mediated 2-5A synthesis (Hovanessian and Justesen, 2007; Minks et al., 1979; Player and Torrence, 1998). As shown in Table 3, the levels of 2-5A in these cells (762–1548 pmol/dL) was sufficiently high as to require dilution in order to obtain values falling within the assay range. These results were not surprising given the ability of dsRNA to both strongly induce expression of OAS and activate the different isoforms. The detection of 2-5A by RIA was also applied to lysates from CVB1 or HAV infected cells (Table 3). 2-5A was reproducibly detected in independent extracts from CVB1 infected FRhK-4 cells (31–35 pmol/dL, 10 h pi). Analysis of diluted CVB1 lysates gave similar 2-5A values, again supporting the reproducibility of the analysis. Similar levels of 2-5A were detected in HAV/18f infected cells at 24 h pi (33–63 pmol/dL) with an increase in levels observed with increasing time of infection (202 and 137 pmol/dL at 48 h pi and 72 h pi, respectively). Most importantly, the detection of 2-5A in CVB1 and HAV/18f infected cells is consistent with the detection of rRNA degradation, and therefore RNase L activation, observed in these infected cells (Figs. 1C and D). 2-5A was not detected, however, in persistently HAV infected clone 1 cells in which activation of RNase L does not occur. Therefore, in FRhK-4 cells activation of RNase L appears to correlate with the synthesis of 2-5A. In summary, the data in Figs. 8 and 9 indicate that both CVB1 and HAV/18f infection are capable of inducing endogenous OAS enzymatic activity in FRhK-4 cells resulting in the production of authentic 2-5A, the activator of RNase L activity observed in these infected cells.

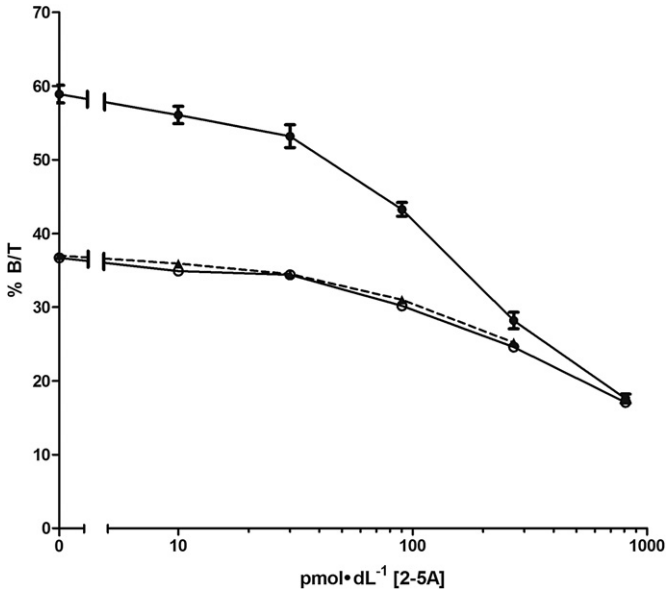

Fig. 9. Plot of binding ratio versus concentration of 2-5A: graphical comparisons of the 2-5A standard curve and the effect of control FRhK-4 lysates. (●—●) The plot of percent bound cpm/total cpm (%B/T) for 0–810 pmol/dL 2-5A standards as the average (± standard error of the mean) of three independently obtained standard curves. Performance characteristics as defined by the manufacturer were determined (and met) for each RIA. Theoretical (▲---▲) and experimental (○---○) binding curves demonstrating the effect of control, undiluted lysate on the analysis of samples containing known quantities of 2-5A. Refer to Materials and methods section for details regarding Fig. 9. For all plots the % B/T equals cpm sample/total cpm × 100.

Discussion

The RNase L-mediated cleavage of cellular and viral RNAs is regarded as an important IFN-regulated antiviral mechanism that restricts virus replication (particularly picornaviruses) whereby viral dsRNA can function both as an inducer of IFN expression as well as an activator of some interferon-stimulated genes (e.g., OAS) that are critical for downstream activation of RNase L (Jacobs and Langland, 1996; Player and Torrence, 1998; Samuel, 2001; Sarkar and Sen, 2004). The results from previous studies in our laboratory revealed an apparent activation of RNase L (viz. RNA degradation) during HAV (cytopathic strain 18f) infection without a requirement for interferon pretreatment, and in the absence of detectable OAS1 or OAS2 mRNA expression (Goswami et al., 2004; Kulka et al., 2003). Although the latter analyses had not been completed for CVB1 infected cells, it seemed an interesting possibility that similar events may be occurring during infection with this virus. In other words, despite the contrasting phenotype of these two viruses, our results suggested that both viruses were capable of successful replication in apparent contradiction to the reported sensitivity of picornaviruses to the 2-5OAS/RNase L antiviral pathway (Bisbal and Silverman, 2007; Jacobs and Langland, 1996; Player and Torrence, 1998; Samuel, 2001). In the current investigation, therefore, we sought to determine the relationship of 2-5OAS expression, IFN-dependence, and 2-5A (the activator of RNase L) synthesis to the activation of the 2-5OAS/RNase L antiviral pathway during CVB1 infection, thereby determining whether or not this pathway lacks the functionality as an antiviral mechanism against CVB1 infection as was previously reported for HAV/18f infected cells. To facilitate our analysis, it was necessary to develop the tools appropriate for a more in-depth investigation in the monkey kidney cell line FRhK-4, such as i) generating affinity purified anti-OAS antibodies for detection of OAS protein isoforms 1–3, ii) designing new PCR primers for targeted amplification of rhesus monkey OAS mRNA isoforms 1–3 (Tables 1 and 2), and iii) adapting existing RIA technology to the detection of 2-5A in lysates derived from mock, infected or dsRNA treated cells (Fig. 9 and Table 3).

It is well known that dsRNA plays an important role in the induction of antiviral pathways such as through activation of the IFN-regulated 2-5OAS/RNase L and the PKR pathways, as well as induction of IFN synthesis (Gribaudo et al., 1991; Jacobs and Langland, 1996; Marie et al., 1990; Minks et al., 1979; Nilsen et al., 1981; Player and Torrence, 1998). We demonstrated that both CVB1 and HAV/18f are capable of generating viral dsRNA due to the presence of positive and negative strand viral genomic RNA generated during replication (Fig. 6). In addition, RNase L was readily detected in CVB1 and HAV/18f infected cells, as well as dsRNA transfected cells (Fig. 2). Not surprisingly, therefore, we found that infection with CVB1 or HAV/18f activates the RNase L pathway in FRhK-4 cells in a manner

equivalent to that observed for dsRNA treated cells (Fig. 1). However, activation of endogenous, latent RNase L requires 2-5A synthesized from dsRNA-activated 2-5OAS, and previous studies revealed a paucity of OAS1 and OAS2 expression in FRhK-4 cells (Goswami et al., 2004).

In the current investigation we conducted a rigorous investigation of the expression of mRNA and protein of all three 2-5OAS isoforms (Figs. 3 and 4) in either CVB1 or HAV infected, or IFNβ treated FRhK-4 cells by i) RT-PCR using PCR primers designed to specifically target rhesus monkey cDNA sequences corresponding to each of the three 2-5OAS isoforms (Table 2), and ii) western blot analysis using affinity purified rabbit antiserum capable of detecting all three isoforms of 2-5OAS protein (Chebath et al., 1987a; Table 1). We found that FRhK-4 cells are not inherently defective for synthesis of 2-5OAS since there is an increase in the level of all three isoforms of 2-5OAS mRNAs following 2 and 12 h IFNβ treatment. However, an increase in the level of OAS2 and OAS3 proteins is observed only at 12 h IFNβ post-treatment. The level of OAS1 protein post-IFNβ treatment did not increase at all to a level sufficient for detection by western blot analysis. Importantly, analysis of OAS expression following either CVB1 or HAV/18f infection revealed an absence of induction in the level of OAS1 or 2 proteins or their respective mRNAs. In contrast, OAS3 mRNA and protein were observed in both uninfected and infected cells indicating that this isoform is constitutively expressed in FRhK-4 cells and continues to be present throughout infection with either CVB1 or HAV. It has been previously reported that cellular levels of individual 2-5OAS isoforms can vary among cell lines and these levels can change in response to either IFN treatment or virus infection (Chebath et al., 1987a, 1987b; Flodstrom-Tullberg et al., 2005; Geiss et al., 2001; Hovanessian et al., 1987, 1988; Rebouillat et al., 2000; Takahashi et al., 2002; Witt et al., 1993; Zhu et al., 1998). However, the predominance of OAS3 expression over OAS isoforms 1 and 2 in FRhK-4 cells, particularly in the context of RNase L activation by CVB1 and HAV/18f, was intriguing given that the contribution of OAS3 to activation of RNase L (viz. 2-5A synthesis) is currently unknown.

In order to investigate whether endogenous OAS levels are enzymatically functional (i.e., activated by dsRNA), cell extracts from virus infected or IFN-treated cells were used in an *in vitro* assay. 2-5A product formation was analyzed by urea-PAGE as previously described (Miele et al., 1991; Sarkar and Sen, 1998) and compared to products generated *in vitro* from rabbit reticulocyte lysate (RL) which is known to synthesize authentic 2-5A in response to dsRNA (Fig. 8). The results of our analyses indicate that the products synthesized by reticulocyte lysate (RL), IFN-treated cell extracts and virus infected cell extracts were qualitatively identical in nature after alkaline phosphatase and RNase 1 digestion. Thus, the synthesized products contain both an RNase resistant 2′-5′ linkage as well as phosphatase sensitive terminal phosphates and represent authentic 2-5A. The differences in the relative size and amount of oligomeric species generated from IFN-treated versus virus infected cells are likely due to the elevated levels of all three OAS isoforms following interferon treatment in FRhK-4 cells (Figs. 3A and 4B) compared to the lower levels of the single isoform OAS3 present in CVB1 or HAV/18f infected cells (Fig. 3, panels C and D; Fig. 4, panels C and D). These differences are consistent with previous reports indicating that OAS2, which we detected in the IFN-treated but not virus infected FRhK-4 cells, is capable of synthesizing a greater abundance of longer 2-5A species (Hovanessian and Justesen, 2007; Marie et al., 1997; Sarkar and Sen, 1998). Investigators have questioned the role of OAS3 in the activation of RNase L due to its synthesis primarily of 2-5 dimers that do not activate RNase L (Hovanessian and Justesen, 2007; Sarkar and Sen, 2004). However, activated OAS3 does not exclusively synthesize 2-5A dimers, as the range of oligomer size has been shown to be from 2 to 10 bases (Hovanessian et al., 1988; Marie et al., 1997; Rebouillat et al., 1999; Sarkar and Sen, 1998). Indeed, in our study OAS3 is essentially the only isoform detected concomitant with the *in vitro* production of a range of 2-5A species from either CVB1 or HAV/18f infected cells (Fig. 8). Furthermore, the analysis of endogenous 2-5A (by RIA) in extracts from these infected cells also reveals the synthesis of authentic 2-5A (Table 3). Given that activation of RNase L is observed in these infected cells and 2-5A dimer is not an activator of RNase L, it is reasonable to conclude that synthesis of active 2-5A (i.e., trimer and larger) is occurring during CVB1 and HAV/18f infection and OAS3 is the likely generator of these active species. These results are significant as we are unaware of any published reports of an activated 2-5OAS/RNase L pathway in the presence of only OAS3 expression.

At present, we are unable to explain why CVB1 infected cells contain lower levels of 2-5A and synthesize 2-5A at a lower rate *in vitro*, compared to HAV infected cells (Table 3; Fig. 8), although RNA cleavage is readily apparent. One reason could be the general status of CVB1 infected cells, compared to HAV infected cells, particularly in regard to the percentage of cells undergoing cpe (cytopathic effect)/apoptosis and shut-off of host protein synthesis. At 8 h pi CVB1 infected cells show 100% cpe as opposed to only 10–20% cpe in HAV/18f infected cells after 48 h. Also, virus-mediated host (protein) shut-off occurs in CVB1 but not HAV/18f infected cells. The result of either process could affect a reduction in the level of functional 2-5OAS available for induction of 2-5A synthesis. Indeed, we observed a reduction in the level of detectable OAS3 following CVB1 infection compared to either HAV/18f infected or IFNβ treated FRhK-4 cells (Fig. 3). Alternatively, the activity of an endogenous 2′-5′ phosphodiesterase (Kubota et al., 2004), a potential regulator of intracellular 2-5A levels, may be elevated following CVB1 infection.

FRhK-4 cells are permissive for CVB1 and HAV/18f replication and these viruses can be grown to high titers in this cell line (Kulka et al., 2003; Goswami et al., 2004). It is currently unknown why their replication appears unabated in these cells despite the activation of the antiviral pathway 2-5OAS/RNase L. Perhaps in FRhK-4 cells RNase L is sufficiently activated to initiate rRNA cleavage but cleavage of viral genomic RNA may be restricted due to its protective interaction with viral/host proteins. Alternatively, 2-5OAS/RNase L pathway activation, as measured by onset of rRNA degradation (>7–8 h pi for CVB1; ≥16 h pi for HAV/18f), may occur too late in the replication cycle to have any significant effect on viral RNA integrity. Ultimately, we have demonstrated that these picornaviruses are not inhibited by an activated 2-5OAS/RNase L antiviral pathway suggesting that picornavirus sensitivity might be species dependent. We have not investigated whether other antiviral mechanisms, such as the dsRNA-activated protein kinase (PKR) or RNA editing, are activated during either CVB1 or HAV/18f infection in these cells. Many of these mechanisms, however, are generally regarded as IFN-inducible (Samuel, 2001; Sarkar and Sen, 2004). In this regard, we have no indication that IFN is produced during CVB1 infection (Figs. 5A and B) and IFN pretreatment has no apparent effect on virus replication or viral protein expression (Fig. 7B). This suggests that the other IFN-regulated antiviral mechanisms are either not activated, or CVB1 and HAV/18f replication are unaffected by their activity. Alternatively, it remains a possibility that activation of other antiviral mechanisms may occur late in CVB1 infection in the absence of IFN. If this were true, however, then one would conclude that a putative late activation of another antiviral pathway also has little relevance in limiting the replication of these viruses.

We do not know why HAV/HM175 clone 1 is unable to activate the 2-5OAS/RNase L pathway even after several months of persistent infection (Fig. 1). There is no indication of any unusual processing of RNase L protein when comparing dsRNA treatment to infection with either CVB1 or HAV (18f or clone 1) in FRhK-4 cells (Fig. 2) and OAS3 protein is constitutively expressed at levels similar to those detected in uninfected FRhK-4 cells (Fig. 3). The potential for viral dsRNA formation exists in both CVB1 and HAV infected cells since viral negative strand RNA is detected by strand specific RT-PCR (Figs. 6, panels A and B), yet, only in CVB1 and HAV/18f infected are both 2-5A synthesis and RNase L activation readily detected. Perhaps cellular or

viral inhibitors of RNase L play a role in blocking its activity in clone 1 infected cells (Bisbal and Silverman, 2007; Han et al., 2007; Samuel, 2001; Townsend et al., 2008). Alternatively, we have observed that the lack of detectable 2-5A synthesis/2-5OAS activation (Table 3) in clone 1 infected cells correlates with low levels of viral negative strand RNA [e.g., at 28 days pi (Fig. 6A)], in contrast to the relatively higher levels of viral negative strand RNA and 2-5OAS activation observed during either CVB1 or HAV/18f infection. In addition, the onset of RNase L activity (viz. rRNA degradation) correlates with the virus replication rates where CVB1 is the fastest, and clone 1 is the slowest, of the three viruses tested. Therefore, viral dsRNA levels during clone 1 infection may simply be insufficient to activate any OAS present. It is interesting, however, that the activation parameters of OAS3 have been shown to be different from that of OAS2, at least *in vitro* (Hovanessian and Justesen, 2007; Hovanessian et al., 1988, 1987; Marie et al., 1990, 1997; Player and Torrence, 1998; Sarkar and Sen, 1998). For example, these two isoforms differ in their concentration dependence for minimal and maximal activity whereby OAS3 requires about 10 to 100-fold lower concentrations of dsRNA than OAS2 (Hovanessian et al., 1988; Marie et al., 1997; Rebouillat et al., 1999; Sarkar and Sen, 1998). If a similar dsRNA concentration dependence for OAS activation occurs *in vivo*, then the contribution of OAS3 to the activation of RNase L might be unapparent in cells infected with rapidly replicating picornaviruses, such as EMC, Mengo, poliovirus or CV, when OAS2 and/or OAS1 are present. As suggested by the results of this investigation, we believe the biological relevance of OAS3 resides in its potential for activation by slower replicating picornaviruses such as HAV/18f.

FRhK-4 cells are functionally responsive to either IFNα or IFNβ treatment (Figs. 3 and 4; Goswami et al., 2004; Kulka et al., 2003) yet we have observed that pretreatment with IFNβ does not inhibit either virus replication (at least at high moi) or viral protein synthesis (Fig. 7), nor does it augment RNase L activity in CVB1 infected (Fig. 7) or HAV infected cells (Goswami et al., 2004). This is in contrast to previous reports on the augmented activation of the RNase L pathway and subsequent inhibition of EMC virus (a picornavirus) growth and following IFN pretreatment (Cayley et al., 1982; Silverman et al., 1982). Given that onset of rRNA degradation is fairly rapid during CVB1 infection; we interpret our results to suggest that sufficient 2-5A is already synthesized for maximal activation of RNase L in these cells during CVB1 infection. As for HAV/18f, the onset of rRNA degradation occurs later in infection for this slower growing virus, and viral dsRNA may not reach levels sufficient to activate increased levels of OAS, particularly OAS2 and/or OAS1. For infection with either virus, therefore, any IFN-mediated increase in OAS levels would have little net effect on RNase L activity. We also conclude that there is no significant synthesis of IFNβ in response in either CVB1 or HAV/18f infected FRhK-4 cells (Fig. 5; Goswami et al., 2004; Kulka et al., 2003), and the fact that both viruses replicate to high titer in these cells whether infected at low or high moi is consistent with the absence of IFN synthesis. It is possible that virus-mediated mechanism(s) responsible for the inhibition of IFN synthesis in other picornavirus infected cells may also exist in CVB1 infected FRhK-4 cells (Barral et al., 2007; Brack et al., 2002; Fensterl et al., 2005; Goswami et al., 2004; Yang et al., 2007). In particular, the proteosome-/caspase-dependent cleavage of MDA5 suggested as responsible for the absence of interferon synthesis following infection with either poliovirus or rhinovirus 1a (Barral et al., 2007) might be relevant for CVB1 and HAV/18f since infection with these viruses activates the caspase-mediated apoptotic pathway in FRhK-4 cells (Goswami et al., 2004; Goswami and Kulka, unpublished).

The activation of RNase L has been implicated in the induction of apoptosis (Castelli et al., 1988; Rusch et al., 2000; Zhou et al., 1997), although the mechanisms by which the two processes are connected are not well understood. Based on the appearance of caspase-3 and PARP cleavage, TUNEL positive cells, and cytoplasmic DNA laddering, we concluded that the cytopathogenicity during HAV/18f infection in FRhK-4 cells was due to the induction of apoptosis by a mechanism involving activation of RNase L (Goswami et al., 2004). Interestingly, we also obtained similar results following CVB1 infection in these cells (Goswami and Kulka, unpublished). It seems plausible that activation of rRNA cleavage and induction of apoptosis may either be necessary for facilitating CVB1 and/or HAV/18f replication in FRhK-4 cells, or alternatively these cellular processes may be inhibited until the virus (es) can complete their replication cycle due to the potential for detrimental effects on replication. Indeed, either scenario seems likely since activation of RNase L occurs late in the respective life-cycles of these viruses (7–8 h pi for CVB1; 16–24 h pi for HAV/18f) and growth of these two viruses continues before apoptosis is induced (8–10 h pi for CVB1; 48 h pi for HAV/18f). The relationship between apoptosis and the growth/replication of CVB1 and HAV in permissive cells continues to be the focus of ongoing research in our laboratory.

Since we can detect the expression of 2-5OAS, the activation of endogenous OAS (with dsRNA) with synthesis of authentic 2-5A, and the presence of endogenous cellular 2-5A concomitant with activation of RNase L during either CVB1 or HAV/18f infection in FRhK-4 cells, it is reasonable to conclude that the 2-5OAS/RNase L pathway is activated in these cells. Our results also indicate that i) the 2-5OAS/RNase L pathway plays a minimal, if any, role in the inhibition of CVB1 or HAV/18f replication, and ii) IFN may play only a limited role as an antiviral compound in FRhK-4 cells. Most importantly, these results suggest that constitutive levels of OAS, in particular OAS3, may be sufficient for synthesis of 2-5A and activation of RNase L in FRhK-4 cells during virus infection, although not all virus infections (e.g., HAV/HM175 clone 1) will trigger this response. Our results suggest a need for further investigation into picornavirus replication/dsRNA formation and OAS3 activation in FRhK-4 cells compared to other monkey and human kidney cell lines.

Materials and methods

Cells and viruses

Coxsackievirus B1 strain Conn-5 (CVB1), HAV strain 18f (HAV/18f) and HAV HM175/clone 1 (clone 1) were obtained from ATCC (Manassas, VA). The 18f strain of HAV is a rapidly replicating apoptosis inducing variant, originally derived from a cell culture adapted wild-type strain HM175 (Cromeans et al., 1989; Lemon et al., 1991) while the clone 1 strain of HAV is a non-cytopathic cell culture adapted strain derived from wild-type HM175 (Daemer et al., 1981). The FRhK-4 (fetal rhesus monkey kidney) cell line was obtained from Dr. G. Kaplan (Center for Biologics Evaluation and Research, FDA) and cultured in MEM supplemented with pyruvate and non-essential amino acids (Invitrogen, Gibco Cell Products, Carlsbad, CA) containing 5% heat-inactivated fetal bovine serum. The 293 (human kidney) cell line purchased from ATCC was cultured in complete MEM (as above) except with 10% heat-inactivated fetal bovine serum. HAV stocks were prepared in FRhK-4 cells essentially as described by Dotzauer et al. (2000), and CVB1 stocks were prepared in FRhK-4 cells based on the method described for CVB3 by Zaragoza et al. (1997) using a 14–16 h incubation period. The establishment of persistently HAV HM175/clone 1 infected cells, as well as procedures for acute infection with CVB1, 18f, and clone 1 virus and virus titration have been described previously (Kulka et al., 2003; Goswami et al., 2004).

Antibodies

Mouse monoclonal antibody (mAb) to human RNase L was obtained as a generous gift from Dr. R. H. Silverman (Cleveland Clinic and Foundation, OH) for use in initial analyses and subsequently obtained from Abcam (Cambridge, MA). In western blot analysis, this mAb detects human (Rusch et al., 2000) and monkey (Goswami, B.

and Kulka, M., unpublished observation) RNase L. Rabbit anti-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-phospho^{Y701}-STAT-1 and rabbit anti-STAT-1 were purchased from Cell Signaling Technologies (Beverly, MA). Coxsackievirus B1 (horse) antiserum was purchased from ATCC (Manassas, VA) that reacts with a 33 kDa (apparent molecular weight) protein from CVB1, but not mock, infected cell extracts (Fig. 1A) consistent with the size of CVB virus capsid protein VP1 (Haarmann et al., 1994); it is not cross-reactive with HAV proteins in western blot analysis (data not shown). Polyclonal rabbit antiserum to HAV capsid VP1 has been previously described by Kulka et al. (2003). Goat anti-rabbit and goat anti-mouse horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Pierce Biotechnology Inc. (Rockford, IL) and goat anti-horse HRP-labeled secondary antibody was purchased from Rockland, Inc (Gilbertsville, PA). Polyclonal antibody to human 2-5OAS was obtained from New Zealand white rabbits immunized with a synthetic peptide CEKYLRRQLTKPRPVILDPAD. This peptide sequence was based on peptide B described by Chebath et al. (1987a). The N-terminal C (cysteine) was included in the peptide sequence to facilitate coupling to a carrier protein KLH (keyhole limpet hemocyanin). In Table 1, a comparison of amino acid sequences within each OAS isoform homologous to the immunizing peptide is listed for both human and rhesus monkey 2-5OAS proteins. The immunizing peptide incorporates the conserved N-terminal amino acids of exon E of human 2-5OAS and was shown to be capable of detecting differential expression of all three isoforms of 2-5OAS among a variety of cell lines (Chebath et al., 1987a). The peptide was synthesized at FDA (Division of Virulence Assessment, CFSAN) and KLH-conjugated using the Imject Maleimide Activated mcKLH Kit (Pierce) according to manufacturer's instruction. Collection of pre-immune serum, immunizations and challenges with KLH-conjugated peptide in adjuvant, hyperimmunization with free peptide and immune serum collection were as previously described (Kulka et al., 2003). All animal studies were conducted in compliance with the Guide for Care and Use of Laboratory Animals under an approved IACUC protocol. Preliminary studies using hyperimmune anti-2-5OAS whole antiserum against dsRNA treated or control FRhK-4 cell protein extracts in western blot analysis revealed a strong induction of OAS2 and OAS3 that follows dsRNA treatment. In the absence of dsRNA treatment, only OAS3 was detected, albeit at low levels (data not shown). Results of western blot analysis were completed following purification of this polyclonal serum and revealed a significant improvement in the sensitivity of OAS detection (data not shown). Affinity purified antiserum was therefore used in all subsequent analyses presented in the current investigation. Purification of anti-2-5OAS antiserum by affinity chromatography was accomplished using immunizing peptide coupled-agarose (SulfoLink Kit, Pierce) according to manufacturer's instructions.

Virus infection and IFNβ treatment

FRhK-4 cells were mock, HAV/18f or CVB1 infected at an moi of 8–10 (60 or 90 min adsorption for CVB1 or HAV/18f, respectively) for the indicated times at 37 °C in growth media containing 1% heat-inactivated FBS. Cell cultures were harvested by scraping, and washed (cold PBS) cell pellets were obtained for subsequent nucleic acid, protein or 2-5A extraction (Kulka et al., 2003; Goswami et al., 2004). Persistently HAV HM175/clone 1 infected FRhK-4 cells were continuously cultured (Kulka et al., 2003; Goswami et al., 2004) and used to obtain cell pellets (100–150 days post-infection [pi]) for nucleic acid, protein or 2-5A extraction as described for mock and acutely infected cells. Routinely cultured FRhK-4 cells were the source of uninfected, non-treated cell pellets and extracts described in this study. Interferon-β (Sigma Chemicals, St. Louis, MO) treatment (1000 U/ml) was added to cell cultures in complete growth media and cells were incubated for the times indicated.

Double-stranded (ds) RNA transfection

FRhK-4 cells were transfected with 25 μg/ml dsRNA [poly(I):poly (C), Amersham] using 20 μg/ml lipofectin (Invitrogen) based on manufacturer's protocol for transfection with DNA. Parallel mock transfections (i.e., without dsRNA) served as negative controls. Cells were harvested at 24 h post-transfection for subsequent extraction of RNA or protein isolation, or 2-5A.

RNA isolation, rRNA degradation analysis, and protein extracts

Total cytoplasmic RNA was isolated for the analysis of rRNA degradation by agarose gel electrophoresis as described previously (Kulka et al., 2003; Goswami et al., 2004). Similarly extracted RNA was also used for the analysis of 2-5OAS, RNase L or IFNβ mRNA expression by RT-PCR. RNA concentrations were calculated from the absorbance measured at 260 nm. Protein extracts were obtained as total cell (soluble) lysates from mock or virus infected cells using RIPA buffer (plus protease and phosphatase inhibitors) as previously described (Kulka et al., 2003). Fractions were stored in aliquots at −70 °C. Protein concentrations were determined using the BCA-200 Protein Assay (Pierce) according to the manufacturer's instructions.

Reverse transcription and PCR

Total cytoplasmic RNAs were subjected to DNase I digestion, phenol:chloroform:isoamyl alcohol (Invitrogen) extraction and ethanol precipitation prior to reverse transcription (1 μg RNA per reaction) with AMV reverse transcriptase (RT) using an oligo(dT)$_{15}$ primer (2 μg per reaction) as previously described (Goswami et al., 1994, 2004). For PCR amplification of OAS isoforms, 4 μl of the cDNA preparation was amplified using Taq polymerase (Promega) in a 50 μl PCR reaction containing 150 μM dNTPs with 50 pmol of each (sense and antisense) primer, cycling parameters and optimal magnesium chloride concentrations as indicated in Table 2. PCR amplification of RNase L was completed as described for OAS except using an annealing temperature of 60 °C, a 180s [72 °C] extension, 2.5 mM $MgCl_2$, for 30 cycles, and 5′ CACCATGGAGAGCAGGGATCATAAC 3′ and 5′ TGAACTCCAGCAAATC AGTCC 3′ as the sense and antisense primers, respectively. These primers target nucleotide sequences which flank the entire coding region of RNase L and share identity with both the human (Genbank accession L10381) and Rhesus monkey (Genbank accession EF467993) sequences to generate a 2.2 kb amplicon from either species. Following RT, PCR amplification of IFNβ (35 cycles) using a primer pair specific for IFNβ at an annealing temperature of 56 °C was based on Goswami et al., 2004 to generate a 552 bp amplicon (Brack et al., 2002) and of β-actin (2.5 mM $MgCl_2$, 30 cycles) using a commercially available primer pair (Promega) was based on cycling conditions described for OAS except using an annealing temperature of 65 °C to generate a 285 bp amplicon. Except where noted, all PCR primers were obtained from Loftstrand Labs, Ltd (Gaithersburg, MD). PCR products were analyzed by agarose gel electrophoresis (Goswami et al., 2004). The identities of amplified OAS and RNase L were confirmed by sequence analysis (Amplicon Express, Pullman, WA).

Strand selective RT was used to demonstrate the presence of viral positive and negative strand RNA (Chehadeh et al., 2000). The following primer sequences were used for these experiments: HAV sense: 5′ CCGTTTGCCTAGGCTATAGGCTA 3′; HAV antisense: 5′ CAGC-TCCATGCTAATCATGGAGT 3′; CVB1 sense: 5′ CCCCTGAATGCGGC-TAATCC 3′; CVB1 antisense: 5′ GCCAMTCCAATARCTATATG 3′, where M and R represent A or C, and A or G, respectively.

PAGE and Western blot analyses

Equal amounts of protein (RIPA) extracts were adjusted to equivalent volumes in denaturing sample buffer containing 100 mM

DTT, heated at 95–98 °C for 5–10 min and subjected to SDS-PAGE [4–12% Bis–Tris gels using MOPS-SDS running buffer (Invitrogen)] under reducing conditions, followed by electrophoretic transfer onto nitrocellulose in 0.65×Transfer Buffer (Invitrogen) containing 10% methanol. Western blot analysis was performed as previously described for antibodies to HAV capsid protein VP1, and actin (Kulka et al., 2003; Goswami et al., 2004). Primary antibodies were diluted (anti-CVB1 at 1:1500; anti-VP1 at 1:1400; anti-OAS at 1:270; anti-actin at 1:1750; anti-RNaseL at 1:1000) in TBS-T containing 5% nonfat dry milk [NFDM] or 5% BSA (anti-STAT and anti-phospho^{Y701}-STAT1 at 1:1000) for overnight incubation at 4 °C. Secondary antibody-HRP conjugates were used at a 1:10,000 dilution in TBS-T/5% NFDM for 1 h incubation at RT and subsequent detection by chemiluminescence was as previously described (Kulka et al., 2003).

^{35}S-labeling and SDS-PAGE

FRhK-4 cells were grown in 25 cm^2 flasks and 12–14 h prior to virus infection treated or not with growth media containing 1000 U/ml human IFNβ. Cells were infected with CVB1 as described above under "virus infection" and labeled for 2 h beginning at 6 h pi with 50 μCi/ml of ^{35}S-methionine (GE Life Sciences) in methionine-free medium supplemented with 5% dialyzed FBS (ICN) following a 30 min pre-incubation in methionine-free medium. Cells were collected by scraping, washed once with PBS and the pellets lysed in 400 μl RIPA buffer containing protease inhibitors (Sigma-Aldrich). Lysates were clarified by centrifugation (10 k ×*g*, 10 min, 4 °C), 10% TCA precipitable incorporation was measured using 10 μl of clarified lysate diluted 1:10 in RIPA buffer, and protein concentration was determined as described under "RNA and protein extracts." Labeled proteins (200,000 cpm per lane) were subjected to SDS-PAGE as described under "PAGE and Western blot analysis", the gel was fixed and treated with Amplify for signal enhancement according to manufacturer's instructions (Amersham Pharmacia Biotech Inc., Piscataway, NJ), then soaked in water containing 20% ethanol and 10% glycerol for 30 min prior to overnight drying between cellophane sheets (Diversified Biotech, Boston, MA). The dried gel was exposed to a phosphorimager screen (4–10 days) then scanned using the Typhoon scanner (Molecular Dynamics Inc., Sunnyvale, CA).

In vitro synthesis and analysis of 2-5A

^{32}P-labeled 2-5A was synthesized following the incubation of cell extracts absorbed to poly(I):poly(C)-agarose in the presence of α-^{32}P ATP. 2-5A synthesized from nuclease treated rabbit reticulocyte lysate (Promega) absorbed to poly(I):(C)-agarose was used as standard. Cell extracts were prepared from interferon-β treated (1000 U/ml, 16 h), uninfected or virus infected cell pellets lysed in buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 7.5 mM 2-mercaptoethanol) containing protease inhibitor cocktail (EDTA-free, Pierce) and 0.5% NP40, following mild Dounce homogenization. The lysate was centrifuged at 15,000 ×*g* for 15 min, the supernatant was removed and adjusted to10% in glycerol supplemented buffer and stored at −70 °C.

For synthesis of 2-5A, cell extracts (200 μl, approximately 50 μg protein) were adjusted to 140 mM KCl, 5 mM $MgCl_2$, 7.5 mM 2-mercaptoethanol containing EDTA-free protease inhibitor cocktail for incubation with poly(I):(C)-agarose. Eighty microliters of a 50% poly(I):poly(C)-agarose suspension, washed thoroughly with a solution of 20 mM HEPES, pH 7.4,140 mM KCl, 5 mM $MgCl_2$ and 7.5 mM 2-mercaptoethanol, was used to obtain a 40 μl (packed) agarose pellet for resuspension with 200 μl of cell extracts. The suspension was mixed gently at room temperature for 15 min, centrifuged and the packed resin washed three to four times with the binding buffer to remove unbound proteins. The washed pellets were incubated with equal volumes of a reaction mixture containing 40 mM HEPES, 20 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 2 mM ATP, 20% glycerol, and 2 μl ^{32}P labeled ATP (GE Amersham, 3000 Ci/mMol, 10 mCi/ml). Rabbit reticulocyte lysate was diluted with an equal volume of the binding buffer and 200 μl (0.5 to 1 mg protein) was used for each 40 μl packed volume of the poly(I):poly(C)-agarose. The washed pellets were incubated as described above for cell extracts. After overnight incubation at 30 °C, the reactions were stopped by heating at 95 °C for 5 min, and centrifuged. Reaction products (2 μl) were analyzed by 7 M urea, 20% polyacrylamide gel electrophoresis essentially as described by Miele et al. (1991) with or without treatment with bacterial alkaline phosphatase (TaKaRa, 0.4 U/μl). The gel was exposed to a phosphorimaging screen after removing the glass plate from one side, and wrapping in Saran wrap while still on the opposing glass plate, and the screen was scanned in a Typhoon 8600 scanner.

Analysis of cellular 2-5A levels by radioimmunoassay (RIA)

Soluble cell lysates were prepared from FRhK-4 cells grown in 75 cm^2 flasks for analysis of endogenous levels of 2-5A. Duplicate culture flasks were mock, CVB1 or HAV/18f infected, or control or dsRNA transfected, for the indicated times to obtain pooled, washed pellets. Routinely sub-cultured and persistently HAV HM175/clone 1 infected FRhK-4 cell pellets were similarly obtained from duplicate culture flasks. Briefly, cell (150 cm^2 equivalent) pellets were resuspended in 300 μl cold DNase/RNase free water (Gibco Life Technologies, Carlsbad, CA), heated to 98 °C for 15–20 min, immediately chilled in an ice-water bath for 10 min, and centrifuged (4 °C, 40 min, 20K ×*g*) to obtain clarified soluble cell lysates. Following 30 min (minimum) freezing at −70 °C, lysates were lyophilized to dryness (and stored at −70 °C when necessary) prior to resuspension in the 0 pmol 2-5A standard immediately before analysis of 2-5A levels by RIA.

Analysis of 2-5A levels was accomplished using the Synthetase (2-5A) RIA kit (ALPCO Diagnostics, Salem, NH) adapted to directly measure 2-5A in heat-denatured clarified cell lysates prepared as described above. 2-5A present in the resuspended cell extracts or synthesized by poly (I):(C)-activated serum OAS enzymes (positive control; synthetase activity provided by manufacturer) is measured following the addition of I^{125}-2-5A for competitive binding to anti-2-5A antibody and immune-complex formation with secondary antibody. A standard curve is generated using 0–810 pmol authentic 2-5A standards added to post-poly (I):(C)-activated negative control serum samples (serum lacking any synthetase activity). Similarly, resuspended lysates are added to post-poly (I):(C)-activated negative control sera. The antibodies, control sera, cold (standards) and radiolabeled 2-5A are supplied with the kit. The 2-5A standards (10–810 pmol/dL) contain the triphosphate 2-5A trimer and are also provided with the kit. The anti-2-5A serum reacts most efficiently with the 5′ triphoshorylated trimeric and tetrameric forms of 2-5A and with significantly lower binding efficiencies for the 5′ triphosphate dimer and 5′ monophosphate trimer. It also reacts with non-phosphorylated trimer, albeit with even lower binding efficiencies than for the monophosphate trimer, and very low efficiency of binding for 3′-5′ linked oligomers, ATP, ADP, AMP and adenosine. Representative examples of these binding efficiencies may be found in Sawai et al. (1985).

For each independent assay, a standard curve (plotted as the binding ratio versus 2-5A concentration) was generated using manufacturer provided 2-5A (data not shown). A 2-5OAS (positive) control was also provided by the manufacturer and included in each assay. In addition, to facilitate the determination of lysate concentrations of 2-5A (by extrapolation), the standard curve and positive control also provided a metric for evaluation of assay performance based on the manufacturer's specifications. In summary, all standard curves gave essentially overlapping plots and positive control values

were within the range indicated by the manufacturer. To illustrate assay reproducibility, the % B/T plotted as the average ± standard error of the mean (SEM) (with an SEM range of 0.6–1.6) of all standard curves is shown in Fig. 9 (upper curve). Preliminary studies indicated, however, that control lysate alone (i.e., from untreated, uninfected cell extracts) contributes background binding to the RIA (data not shown). To determine whether this background would interfere with the capability of determining 2-5A levels by this method of analysis, two graphical analyses were completed for comparison of their plot characteristics. First, using the standard curve, a theoretical binding curve was generated where i) the percent B/T obtained for control lysate alone was plotted as 0 pmol/dL 2-5A, and ii) the percent B/T predicted for control lysate with increasing concentrations of 2-5A (10–810 pmol/dL) was determined from the standard curve and plotted against the concentration of 2-5A. Second, an experimental binding curve was generated whereby lyophilized control lysates were resuspended with the 0–810 pmol/dL 2-5 A standards and the percent B/T determined and plotted versus the known input of 2-5A. As shown in Fig. 9 (lower plots), both the theoretical and experimental plots are essentially super-imposable indicating that the lysate milieu i) does not interfere with the capability of determining 2-5A levels by this method of analysis, and ii) can be treated as assay background (i.e., 0 pmol/dL 2-5A) for subtraction from experimental values. However, since the change in slope for the control lysate plot is less than that for the standard curve plot over the 10–30 pmol/dL range, we conservatively assigned 30–810 pmol/dL as the assay range for cell lysate analysis rather than 10–810 pmol/dL as given by the manufacturer. Additional performance characteristics were determined (and met) for each RIA. Based on these results we concluded that the assay is both reliable and reproducible.

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