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RNase L and Double-Stranded RNA-Dependent Protein Kinase Exert Complementary Roles in Islet Cell Defense during Coxsackievirus Infection

Malin Flodström-Tullberg,*† Monica Hultcrantz,† Alexandr Stotland,* Amy Maday,* Devin Tsai,* Cody Fine,* Bryan Williams,‡ Robert Silverman,‡ and Nora Sarvetnick2*  

Coxsackievirus (CV) infections are common in humans and have been etiologically linked to type 1 diabetes (T1D) (1, 2). Members of the CV family have been detected in pancreatic tissue, including β cells, from T1D patients and individuals that succumbed to a CV infection (3–6). CVs infect β cells in vitro, often leading to β cell dysfunction and destruction (6–12). Because these viruses have such dramatic effects on β cell survival in vitro, it may seem surprising that the majority of systemic infections pass without the development of T1D (13). Recent studies have explained this paradox. First, an interesting study by Chehadeh et al. (14) demonstrated that human pancreatic β cells survive an in vitro challenge with CV only in the presence of IFN. Subsequent studies in an animal model showed that an intact β cell response to IFNs is indispensable for islet cell survival in vitro and in vivo and that protection from diabetes following systemic CV infection required an intact islet cell response to IFNs (11, 15). Taken together, these studies suggest that β cells survive a systemic CV infection by responding to IFNs released early during the infection. Importantly, the studies also suggest that islet cell activities directly determine the outcome of an infection with a diabeticogenic virus. Indeed, the efficiency by which β cells mount antiviral defense activities may directly regulate an individual’s risk for developing viral-induced T1D (11, 15). This awareness has accentuated the need for further studies on β cell antiviral defense.

IFNs are produced early following a viral encounter, including infections with picornaviruses (Refs. 16–18, and M. Flodström-Tullberg and N. Sarvetnick, unpublished observation). They activate the host’s antiviral immune response and, therefore, are often critical for host survival. Early during viral exposure, cells at the local site of viral entry rapidly produce and secrete type I IFN (IFN-α, -β, and -ω). The type II family of IFNs, containing a single member, IFN-γ, is elicited at a slightly later stage of infection. This cytokine is secreted by activated NK cells, CD4+ T cells, and CD8+ CTLs (19–21). Members of both IFN families contribute to the host’s antiviral defense by up-regulating MHC I expression, activating NK cells, macrophages, and T cells (19–21). Besides this, the IFNs act in auto-, para-, and endocrine fashions, triggering the transition of uninfected cells into an antiviral state and apoptotic cell death in already infected cells (19–23). The overall goal for these actions is prevention of viral infection, replication, and dissemination.

The antiviral state aims at lowering a cell’s permissiveness to infection. This is commonly achieved by the expression of proteins exhibiting intracellular antiviral activities. For example, RNase L degrades viral and host RNA. This endonuclease is activated by 2-5A oligoadenylates (2-5A) synthesized by a family of IFN-regulated enzymes denoted 2-5A synthetases (2-5AS) (24). The 2-5AS enzymes become activated only in the presence of viral dsRNA intermediates. In addition to 2-5AS, IFNs can induce the expression of proteins involved in intracellular antiviral defense. Specifically, we demonstrate that 2’,5’-oligoadenylate synthetases (2-5AS), RNase L, and dsRNA-dependent protein kinase (PKR) are expressed by pancreatic islet cells and that IFNs (IFN-α and IFN-γ) increase the expression of 2-5AS and PKR, but not RNase L. Moreover, our in vitro studies uncovered that these pathways play important roles in providing unique and complementary antiviral activities that critically regulate the outcome of CV infection. The 2-5AS/RNase L pathway was critical for IFN-α-mediated islet cell resistance from CV serotype B4 (CVB4) infection and replication, whereas an intact PKR pathway was required for efficient IFN-γ-mediated repression of CVB4 infection and replication. Finally, we show that the 2-5AS/RNase L and the PKR pathways play important roles for host survival during a challenge with CVB4. In conclusion, this study has dissected the pathways used by distinct antiviral signals and linked their expression to defense against CVB4. The Journal of Immunology, 2005, 174: 1171–1177.
expression of a dsRNA-dependent protein kinase frequently de-
noted PKR (20, 22, 23, 25). In response to viral dsRNA, this
serine/threonine protein kinase phosphorylates and inactivates ri-
bosomal protein eukaryotic initiation factor 2α resulting in a block
in protein translation. The perturbed protein biosynthesis inhibits
viral replication. Other examples of IFN-regulated proteins with
demonstrated antiviral actions are the Mx family of GTPases and the
inducible form of NO synthase (NO synthase 2) (20).

Whether any particular antiviral pathway is favored for IFN-
induced inhibition of viral replication in pancreatic islet cells is
unknown. In the present study, we tested the hypothesis that IFNs
regulate islet cell permissiveness to CV serotype B4 (CVB4) (24),
a serotype associated with the onset of T1D (1, 2), by inducing the
2-5AS/RNase L and PKR pathways. We demonstrate that pancre-
atic islet cells express RNase L, 2-5AS, and PKR and that the
expression of 2-5AS and PKR is increased following exposure to
both IFN-α and -γ. We uncovered a critical role for both antiviral
pathways in host survival following CVB4 infection. Interestingly,
in our in vitro studies revealed that the 2-5AS/RNase L pathway is
required for IFN-α-mediated islet cell resistance against CVB4
infection. Furthermore, we demonstrate that IFN-γ-mediated re-
pression of CVB4 infection of islet cells requires an intact PKR
pathway.

Materials and Methods

Animal husbandry

C57BL/6J mice were originally obtained from The Jackson Laboratory
or Taconic Farms. RNase L$$\gamma$$, PKR$$\gamma$$, and RNase L$$\gamma$$ × PKR$$\gamma$$ mice
(here denoted DKO mice) were bred and maintained at The Scripps Re-
search Institute or Karolinska Institutet. Because RNase L$$\gamma$$ × PKR$$\gamma$$ mice
were originally bred to C57BL/6J mice obtained from The Jackson Laboratory,
C57BL/6J mice from The Jackson Laboratory were used as wild-type (wt)
controls in experiments involving RNase L$$\gamma$$ × PKR$$\gamma$$ mice. PKR$$\gamma$$ mice
had originally been bred to C57BL/6J mice from Taconic Farms, and wt control
mice for the experiments involving PKR$$\gamma$$ mice were purchased from
Taconic Farms. RNase L$$\gamma$$ and PKR$$\gamma$$ mice were intercrossed to obtain
RNase L$$\gamma$$ × PKR$$\gamma$$ mice. Because wt C57BL/6J mice obtained from
The Jackson Laboratory demonstrated a slightly higher susceptibility to
CVB4 infections than C57BL/6 mice from Taconic Farms (see Fig. 2),
these mice were used for all experiments involving DKO mice. All mice were kept in a specific pathogen-
free environment. The animal experiments were conducted in accordance
with institutional guidelines for animal care and use.

Pancreatic islet isolation and culture

Pancreatic islets were isolated from 8–12 wk-old C57BL/6J mice (The
Jackson Laboratory) and cultured as previously described (27). The islet
preparations were cultured for at least 6 days before experiments were
performed to remove exocrine tissue and immune cells (11).

Semiquantitative RT-PCR analysis

Pancreatic islets (C57BL/6J mice; The Jackson Laboratory) were exposed
to IFN-α (1000 U/ml recombinant murine produced in Escherichia coli, catalog number 407293; Calbiochem), IFN-γ (1000 U/ml recombinant mu-
rine produced by transfected insect cells; catalog number 554587; BD
Biosciences), or vehicle for 6 h. Total RNA was isolated using the RNeasy
kit (Qiagen) according to the manufacturer’s instructions. Using the
SuperScript first-strand synthesis system for RT-PCR (Invitrogen Life Tech-
nologies), the RNA was converted into cDNA. The cDNA, diluted 1:1, 1:5,
and 1:25, was subjected to PCR analysis using the following primers: 2-5AS, forward 5′-CCCCATCTGACATGGATGGTGAG-3′; reverse
5′-GAGCTCATATCTTGCCAGTGG-3′; 2-5G primers amplify murine 2-5AS 1A (Oas1a) and 2-5AS 1G
(Oas1g) mRNA. PCR amplifying 2-5AS and actin were run using one
2-min cycle at 94°C, followed by 33 cycles (94°C/30 s, 58°C/45 s, 72°C/60
s) for 2-5AS, and 24 cycles (94°C/30 s, 58°C/45 s, 70°C/60 s), for 2-5AS and actin
respectively. Finally, the samples were incubated at 70°C for 10 min. Pre-
liminary experiments were performed to establish reaction conditions that
allowed reproducible and reliable amplifications. PCR products were run
on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. The bands
were visualized by UV light and photographed. The images were saved and
analyzed using NIH Image 1.63. The intensities of the 2-5AS bands were
expressed in arbitrary density units. In all experiments, the density units for
2-5AS were normalized to the actin density units.

Western blot analysis

Protein extracts were isolated from pancreatic islets (C57BL/6J mice; The
Jackson Laboratory) exposed to cytokines (IFN-α, 1000 U/ml or IFN-γ,
1000 U/ml; for sources for cytokines, see above) or vehicle for 24 h. Pre-
nvious studies had shown that a 24-h exposure of pancreatic islets to IFN-α
or -γ was sufficient to obtain a robust protection from CVB4-mediated
destruction (11). Equal amounts of proteins were separated under denatur-
ating and reducing conditions on SDS-PAGE and transferred to nitricellu-
lose membranes. The membranes were first incubated with a primary Ab
binding RNase L (a rabbit polyclonal Ab generated using the N terminus of mouse RNase L), and signal detection was accomplished as previously
described (27). The membranes were then stripped for 10 min in strip
buffer (0.5 M acetic acid and 0.5 M NaCl) and incubated with a primary Ab
detecting PKR (Santa Cruz Biotechnology). The signal was detected as
above. Finally, the membranes were stripped and rebotted with a mouse mAb to actin to confirm equal protein loading (ICN Biomedicals).

Band intensities were quantified from nonsaturated exposures using the
NIH Image 1.63 software (program and source code) and expressed in units of density. In all
experiments, the densities of RNase L and PKR were corrected by
values of actin density.

Virus strain and propagation of viral stocks

CVB4 Edwards strain 2 (E2) (26) was originally obtained from C. Gaunt
(University of Texas, San Antonio, TX). A stock of CVB4 was prepared, and
the titer was determined as previously described (30).

Viral infection in vivo and in vitro

Mice aged 8–10 wk were infected with one i.p. injection of CVB4 (100
PFU diluted in 200 μl of HBSS) and sacrificed on days 3 or 4 post infection
(p.i.). Alternatively, the mice were monitored for survival for a 24-day
study period. Pancreatic islets were infected as previously described (11).
Briefly, islets (20 islets per condition) were treated with IFN-α (1000
U/ml), IFN-γ (1000 U/ml), or vehicle for 24 h. The islets were then washed
once in HBSS and infected with CVB4 in 2 ml of HBSS containing 2 ×
10^5 PFU CVB4/ml (2 × 10^5 PFU/μl). After 1.5 h of incubation at 37°C,
the islets were washed three times in HBSS and placed in Millicell culture
plate inserts (Millipore) containing fresh medium (1 ml) and fresh IFN.
The plates were incubated at 37°C, and the medium was changed every second
day for up to 6 days p.i. Fresh IFN was added at each medium change.
Previous experiments had demonstrated that addition of fresh IFN to the
cultures every second day is sufficient to provide islets with maximum
protection from CVB4-mediated destruction (Ref. 11, and M. Flodström-
Tullberg, unpublished data). Viral titers in culture supernatants were de-
termined by a plaque assay (see below).

Virus recovery from infected pancreatic islets and tissue.
determinations of viral titers

The titers of infectious virus in culture medium from infected pancreatic
islets (retrieved every 48 h p.i.) or pancreata retrieved from infected mice
were quantitated by a standard plaque assay technique in HeLa cells (30).
Viral titers were quantitated as PFU per islet, and results were presented as
log_{10} PFU/μl islet. Alternatively, viral titers were quantitated as PFU per gram
of wet tissue and presented in the text as log_{10} PFU per gram of tissue. The
lower detection limit of this assay is 50 PFU/μl islet culture medium
(i.e., 2.5 PFU/μl or 0.4 log_{10} PFU/μl) or 10 PFU/g of tissue.

Ultrastructural analysis of cell death

Infected and control islet cells were subjected to an ultrastructural analysis
by electron microscopy (EM). Islets were fixed in glutaraldehyde (2.5%
gluteraldehyde, 0.1 M sodium cacodylate (pH 7.3), and 1 mM CaCl2) and
processed for Epon/Araldite resin embedding by standard procedures. Ul-
trathin sections were stained with uranyl acetate followed by staining
with Reynold’s lead citrate and examined at the Core Electron Microscope Fac-
ility (The Scripps Research Institute).

Blood glucose determinations

Venous blood glucose concentrations were measured in nonfasting mice
using a Glucometer Elite (Bayer). Animals were considered diabetic if
having a nonfasting blood glucose value >13.8 mM (250 mg/dl) for at least
two consecutive measurements.
Histology and immunohistochemistry

Paraffin sections of formalin-fixed organs were prepared, cut in 5-µm thick sections, and stained with H&E or with a primary Ab against insulin, glucagon (DakoCytomation), or VP-1 (a capsid protein conserved within the members of the enterovirus family; DakoCytomation) biotinylated in-house. Bound insulin and glucagon Abs were detected with a biotinylated secondary Ab (anti-guinea pig IgG or biotinylated anti-rat IgG) in conjunction with the Vectastatin ABC (peroxidase) kit (Vector Laboratories) and the chromogen diaminobenzidine (Sigma-Aldrich). Slides were counterstained in Mayer’s hematoxylin.

Statistical analysis

Results are expressed as means ± SEM. Plaque assay determinations were performed in duplicate, and the mean of the two values was considered as one independent observation. The statistical analyses were performed using Student’s t test (single comparisons), ANOVA (multiple comparisons), or by Kaplan-Meier life table analysis (survival of infected mice).

Results

IFNs increase the expression of 2-5AS and PKR

We asked whether IFNs promote the antiviral state in pancreatic islet cells by inducing the expression of key proteins involved in two intracellular antiviral defense pathways, namely the 2-5AS/RNase L and PKR pathways; we assessed whether IFNs induced the expression of RNase L and PKR in pancreatic islets by Western blot analysis. Due to a paucity of commercially available Abs to 2-5AS, we evaluated the expression of 2-5AS mRNA by RT-PCR analysis.

We determined that islet cells expressed a low basal level of 2-5AS mRNA (Fig. 1, A and B). Following exposure to IFN-α or -γ, the 2-5AS mRNA expression level increased 7- and 4.5-fold, respectively. The cells also expressed RNase L (n = 4; data not shown). However, neither IFN-α nor IFN-γ altered the expression level of this protein (C57BL6, n = 4; data not shown). Finally, we observed that islet cells expressed PKR, extending previous results demonstrating PKR mRNA expression by islets (31). Treatment with IFN-α led to a 2.4-fold increase in PKR expression (Fig. 1, C and D). A similar exposure to IFN-γ resulted in 2.1-fold increase in PKR expression (Fig. 1, C and D). These observations indicate that 2-5AS, RNase L, and PKR are expressed at low basal levels in islet cells. PKR and 2-5AS expression was increased following IFN stimulation, supporting the hypothesis that IFNs induce islet cell expression of proteins participating in antiviral defense.

The 2-5AS/RNase L and PKR pathways are important for host anti-CVB4 defense

The experiments above demonstrated that IFNs induced 2-5AS and PKR expression in islet cells. Therefore, we next asked whether the 2-5AS/RNase L and PKR pathways are important for survival during infection with CVB4. RNase L is the effector molecule downstream of 2-5AS, and a deletion of this gene results in a deficiency in the 2-5AS/RNase L pathway (32). Because the role for RNase L and PKR in host defense during infection with CVB4 was unexplored, we infected mice lacking these genes and monitored them for survival. We found that RNase L−/− mice showed enhanced susceptibility to infection compared with wt mice; although 62% of the wt mice survived the infection, only 7% of the infected RNase L−/− mice survived the 24-day study period (Fig. 2A). Similar to the RNase L−/− mice, animals lacking a functional PKR gene were less resistant to CVB4 infection than their wt controls. Only one (1/21) of the PKR−/− mice survived the initial observation period of 24 days, whereas the majority of the wt mice...
The pancreatic islets comprise 2–3% of the pancreatic tissue and may respond distinctly compared with the bulk pancreatic studies above. We therefore asked whether CVB4 was present in islet cells of infected mice, because this would suggest a functional role for the antiviral pathways in regulating permmissiveness to early CVB4 infection. CVB4 can be visualized in tissue sections using an Ab binding a conserved sequence of CV capsid protein VP-1 (11, 14, 15). We harvested pancreata from infected wt control, RNase L−/−, PKR−/−, and DKO mice on day 3 p.i. The immunohistochemical analysis revealed VP-1-positive cells in exocrine pancreatic tissue of all infected mice (RNase L−/−, n = 3 mice; wt control, n = 3 mice; PKR−/−, n = 3 mice; wt control, n = 3 mice; DKO, n = 3 mice; wt control, n = 6 mice, data not shown). However, none of the mice revealed VP-1-positive cells in their pancreatic islets, which remained intact.

**IFN-α-mediated block in CVB4 replication requires an intact 2-5AS/RNase L pathway**

To address the role for the 2-5AS/RNase L and PKR pathways in islet cell defense, we infected IFN-treated pancreatic islets isolated from RNase L−/−, PKR−/−, and DKO mice with CVB4 and measured islet viability and virus replication.

CVB4 replicated in untreated pancreatic islets from both wt (Fig. 3A) and RNase L−/− (Fig. 3B) mice, and the islets gradually lost their round structure and integrity (as evaluated by light and EM, data not shown). IFN-γ prevented CVB replication in islets from wt mice (Fig. 3A). Interestingly, a similar protection was observed in RNase L−/− islets that had been treated with IFN-γ (Fig. 3B), suggesting that IFN-γ does not use the RNase L pathway to protect islets from CVB4 destruction. Indeed, these islets, as

![FIGURE 3. A–F.](http://www.jimmunol.org/)

**References**

1. ** mean two independent experiments. Original magnification, change on day 2 p.i. On day 4 p.i., the islets were harvested and subjected to EM analysis. Arrows indicate dying islet cells. Images are representative of Laboratory (H11005).

2. ** PANCREATIC β CELL ANTIVIRAL DEFENSE**

3. ** The pancreata of RNase L−/− and PKR−/− mice are permissive to early CVB4 infection**

We next asked whether a lack in the 2-5AS/RNase L or PKR pathways would lead to an early, detectable alteration in pancreatic islet cell permmissiveness to CVB4. In initial experiments, we determined the permmissiveness of RNase L−/− and PKR−/− pancreata for CVB4 infection. This was accomplished by measurements of viral titers in organs harvested from infected mice on day 3 p.i., a time point chosen because it coincides with the peak of CVB4 replication in the murine pancreas (33, 34). Moreover, none of the mice succumbed to the infection before day 3 p.i. The results were as follows (results presented as log_{10} (PFU per gram tissue); mean ± SEM): RNase L−/−, 9.9 ± 0.4 (n = 3 mice); wt control, 9.3 ± 0.2 (n = 2 mice), p = 0.41; PKR−/−, 10.5 ± 0.2 (n = 3 mice); wt control, 10.4 ± 0.1 (n = 3 mice), p = 0.81. These experiments propose that pancreas from RNase L−/− and PKR−/− are permissive to CVB infection and that there is no difference in pancreatic viral load between the infected knockout and wt animals at day 3 p.i.

**FIGURE 3. A–F.** The role for RNase L and PKR in IFN-induced islet antiviral defense. Pancreatic islets were incubated with IFN-α or -γ for 24 h before infection with CVB4. Culture medium was harvested and replaced with fresh medium and IFNs every 48 h for 6 days p.i. Viral titers were measured in the harvested medium. Islets isolated from wt mice (C57BL6J; The Jackson Laboratory) (n = 2 mice) (A); RNase L−/− mice (C57BL6J; The Jackson Laboratory) (n = 2–3 mice) (B); wt mice (C57BL6J; Taconic Farms) (n = 3 mice) (C); PKR−/− mice (C57BL6J; Taconic Farms) (n = 3 mice) (D); wt mice (C57BL6J; The Jackson Laboratory) (n = 2 mice) (E); and DKO mice (C57BL6J; The Jackson Laboratory) (n = 3 mice) (F). *, p < 0.05, **, p < 0.01, and \*, p < 0.001 vs respective untreated controls, ANOVA. The lower detection limit of the plaque assay was 0.4 log_{10} PFU/islet. G–L, IFN-γ fails to prevent islet degradation in the absence of PKR. Electron micrographs showing pancreatic islet cells from wt (C57BL6J; The Jackson Laboratory) (G, I, K) and PKR−/− (C57BL6J; The Jackson Laboratory (H, J, L) mice. The islets were exposed to PBS (I, J) or IFN-γ (G, H, K, L) for 24 h and then mock-infected (G, H), or infected with CVB4 (I–L), washed, and cultured in the absence (I, J) or presence (G, H, K, L) of IFN-γ for 4 days with one medium change on day 2 p.i. On day 4 p.i., the islets were harvested and subjected to EM analysis. Arrows indicate dying islet cells. Images are representative of two independent experiments. Original magnification, ×3900–5200.
well as the IFN-γ-treated wt islets, maintained their round structure for the whole study period demonstrating the protective effect of IFN-γ (data not shown). In contrast, CVB4 replication was not prevented in IFN-α-treated RNase L−/− islets (Fig. 3B), and these islets failed to sustain their integrity. Taken together, these results suggest that IFN-γ regulates permissiveness to CVB4 in an RNase L-independent manner, whereas IFN-α appears to require expression of the 2-5AS/RNase L pathway to efficiently prevent CVB4 replication and islet destruction.

**IFN-γ prevents CVB4 replication by a PKR-dependent mechanism**

We next evaluated whether PKR was important for an intact IFN-induced defense against CVB4. We found no difference in CVB4 replication between wt islets and islets lacking PKR (Fig. 3, C and D). As evaluated by EM, CVB4-infected wt and PKR−/− islets lost their integrity over time, whereas uninfected control and uninfected IFN-treated islets (PKR−/− and wt) remained intact (Fig. 3, G, H, and K, and data not shown). Furthermore, IFN-α afforded complete protection from CVB4 replication (Fig. 3D) and islet destruction (data not shown) in islets from PKR−/− mice. However, CVB4 replication progressed in an unrestricted manner in PKR−/− islets treated with IFN-γ (Fig. 3D) leading to the degradation of these islets (Fig. 3L). From these observations, we conclude that IFN-γ uses the PKR pathway to reduce islet cell permissiveness to CVB4 infection. Moreover, our results demonstrate that IFN-α regulates permissiveness to CVB4 in a PKR-independent manner.

**IFN-induced islet cell antiviral defense is greatly perturbed in the absence of functional 2-5AS/RNase L and PKR pathways**

We isolated and infected islets from DKO mice and their wt controls. In DKO islets (Fig. 3E), the preventative effects of IFN-α or IFN-γ on CVB4 replication were clearly weakened compared with the effects in wt islets (Fig. 3F). Furthermore, IFNs could not prevent the islets from losing their integrity. As evaluated by light microscopy, many islets were disintegrating on day 4 p.i., and on day 6 p.i., most of the islets were completely dispersed into single cells (data not shown). Uninfected islets (DKO and wt islets, untreated or treated with IFNs) maintained their integrity during the study period. Collectively, our observations propose that the 2-5AS/RNase L and PKR antiviral pathways contribute to IFN-induced islet cell defense and that islet cell permissiveness to early CVB4 infection is altered in the absence of both these pathways.

**Discussion**

We tested the hypothesis that the 2-5AS/RNase L and PKR pathways mediate antiviral activities of IFNs in CVB4-infected pancreatic islet cells and made some intriguing observations summarized in Table I. We discovered that RNase L is required for an intact IFN-α-induced defense against CVB4 in vitro. We also unveiled that PKR is indispensable for efficient IFN-γ-induced islet anti-CVB4 defense. That 2-5AS provide resistance to some picornaviruses was demonstrated in the 1980s (35), but its potential role in regulating pancreatic islet cell permissiveness to CVB has not been tested previously. Studies by Bonnevie-Nielsen and colleagues (36, 37) showed that IFN-α increases 2-5AS activity in cell extracts from insulin-producing cell lines and rat islet cells. In the present study, we demonstrate that both IFN-α and -γ increase 2-5AS mRNA expression in islet cells, suggesting that the increased expression level of 2-5AS can at least, in part, explain the observations made by Bonnevie-Nielsen et al.

**Table I. CVB4 replication in IFN-treated pancreatic islet cells lacking proteins involved in antiviral defense**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>RNase L−/−</th>
<th>PKR−/−</th>
<th>DKO</th>
</tr>
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<tbody>
<tr>
<td>IFN-α</td>
<td></td>
<td>+</td>
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<td>IFN-γ</td>
<td></td>
<td>+</td>
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* Compiled from plaque assay data. +, Efficient anti-CVB4 defense, no difference in CVB4 replication when compared with wild-type islet cells treated similarly. −, Impaired anti-CVB4 defense, high levels of CVB4 replication when compared with wild-type islet cells treated similarly. 

Residual anti-CVB4 activity found when compared with untreated (i.e. not treated with IFN-α or IFN-γ) islet cells of same genotype.

Upon activation by dsRNA, the 2-5AS generate 2-5A. The accumulation of 2-5A leads to the activation of RNase L, an enzyme that can regulate viral replication by cleaving viral and cellular RNA (20, 24, 38). Most tissues studied to date express RNase L, and increased expression following IFN stimulation has been reported for some cell types (20, 24). However, until now it has not been known whether islet tissue expresses RNase L and a functional 2-5AS/RNase L pathway. Here we showed that islet cells express RNase L. We also observed that although IFN-α prevented CVB4 replication in wt islets, it failed to successfully restrain CVB4 replication in islets lacking RNase L. Interestingly, the antiviral activity induced by IFN-α correlated with an increased expression of 2-5AS mRNA but not of RNase L, implying that the expression level of 2-5AS, rather than RNase L, is a rate-limiting step for the RNase L-mediated degradation of viral RNA in islet cells. These data are consistent with other reports demonstrating enhanced RNase L activity with increased endogenous levels of 2-5AS (35, 39, 40). Considering that IFN-γ also increased the expression of 2-5AS mRNA, it was surprising to find that the 2-5AS/RNase L pathway does not play an important role in IFN-γ-mediated protection from CVB4. The mechanism(s) underlying this observation remains to be determined.

The present study shows that both IFN-α and -γ can augment PKR protein expression in islet cells. Furthermore, our in vitro infection studies proposed that PKR is a major effector molecule in IFN-γ-induced islet defense against CVB4 in vitro. Similar observations have been reported in other cell types (41). Our studies also indicate that PKR is not used by IFN-α to achieve protection from CVB4 in vitro. Taken together, our observations suggest that the 2-5AS/RNase L and PKR pathways contribute with exclusive and complementary anti-CVB4 signals following exposure to IFN-α and -γ, respectively.

Although RNase L plays an important role in IFN-α-mediated islet cell anti-CVB4 defense in vitro, infections of RNase L−/− mice did not lead to detectable levels of CVB4 protein in the pancreatic islet cells. Moreover, our in vitro studies pointed to an important role for PKR in robust IFN-γ-induced protection of islet cells from CVB4. Still, no CVB4 could be detected in islets from infected PKR−/− mice. These findings showed that islet cell permissiveness to early CVB4 infection in vivo is tightly regulated even if there is a lack in one of these antiviral pathways. Both type I and II IFNs are important mediators of the host antiviral defense and are produced during picornavirus infections (Refs. 18 and 42, and M. Flodström-Tullberg and N. Sarvetnick, unpublished observation). Hence, it is possible that the presence of both types of IFNs ensure that islet cells up-regulate efficient antiviral defenses during CVB4 infection.

Although the anti-CVB4 action of IFN-α and -γ was clearly impaired in islet cells lacking RNase L and PKR, respectively, it
was not completely lost. Weak residual antiviral activities were also observed in IFN-treated islets from DKO mice. Collectively, these observations suggest that other factors besides the 2-5AS/RNase L and PKR pathways may contribute to the regulation of islet permissiveness to CVB4. Indeed, these observations highlight the existence of hitherto undefined IFN-induced anti-CVB4 pathways. DKO mice infected with another member of the picornavirus family, encephalomyocarditis virus, also unveiled antiviral activities beside the ones mediated by PKR and RNase L (43).

Other IFN-induced proteins suggested to exert antiviral activities in infected cells are the Mx protein GTPases, inducible NO synthase, the virus stress-inducible protein p56, and the RNA adenosine deaminase 1 deaminase (19–22, 25, 44). We have ruled out inducible NO synthase as a single determinant for β cell survival during CVB4 infection (33). It is also unlikely that Mx protein activities account for the residual anti-CVB4 activity observed here, because several mouse strains, including the one used here, lack a functional Mx gene (45). However, this does not exclude anti-CVB4 actions by Mx proteins (28) in Mx-positive strains. A more recently described protein is ISG20 (46), and it remains to be determined whether this or other antiviral proteins, such as p56 and RNA adenosine deaminase 1, contribute to the regulation of islet permissiveness to CVB4. In this context, a potential role for the IFNs in altering the expression of viral receptors should not be overlooked.

Aside from playing a role in the antiviral state, PKR, 2-5AS, and RNase L have been implicated in pathways leading to apoptotic cell death (22, 23, 25). IFNs can trigger apoptosis in already infected cells and several recent studies have suggested that 2-5AS, RNase L, and PKR are effector molecules in this death pathway (22, 23, 25). Interestingly, PKR has been shown to mediate islet cell apoptosis induced by poly(I:C) (synthetic dsRNA) or poly(I:C) in combination with IFN-γ in vitro (47). Hence, PKR may be an effector molecule inducing apoptosis under conditions when already infected islets are exposed to IFNs. That IFNs do not normally trigger apoptotic cell death in islet cells (47, 48), despite the constitutive expression of 2-5AS (Refs. 36 and 37, and the present study), RNase L (present study), and PKR (Ref. 47, and the present study), is not surprising, because dsRNA is absent in the uninfected state. In this context, it is noteworthy that our experiments showed that CVB4 replicated equally well in unmanipulated wt islets as in unmanipulated islet from RNase L−/−, PKR−/− or DKO mice. These observations suggest that, at basal expression levels, 2-5AS, RNase L, and PKR do not regulate islet cell permissiveness to CVB4 replication. Similar observations have been reported from other experimental systems (e.g., in Ref. 49).

We found that a lack in either antiviral pathway led to dramatically increased host susceptibility to CVB4. We and others (11, 50) have demonstrated the requirement for an intact host response to IFNs in survival following infection with CV. Therefore, it is possible that the PKR and 2-5AS/RNase L pathways are important effector molecules in the early IFN-mediated antiviral defense. Interestingly, others have demonstrated that a defective host defense in mice lacking the PKR and 2-5AS/RNase L pathways can be attributed both to a defective antiviral defense and to an impaired induction of apoptosis in infected cells (32, 51–53). Although the specific mechanism(s) by which RNase L and PKR provides protection from CVB4-induced death remains to be explored, our observations suggest that both pathways play independent roles for host survival and that one cannot fully compensate for the lack of the other.

Over the years, in vitro studies and animal models have revealed distinct mechanisms to explain how a viral infection can induce autoimmune disease. Although the majority of these studies focused on the self-reactive T cell population, recent studies have suggested that target cell activities may affect the autoimmune process (reviewed in Ref. 54). For example, Yasukawa et al. (55) demonstrated that target cell activities critically contribute to the prevention of myocarditis during CV infection. Our previous studies showed that if β cell antiviral defense fails, then CVB4 will destroy the β cells regardless of other antiviral defense mechanisms that may be mobilized by the host to fight the infection (11, 15). The unraveling of mechanism(s) behind islet cell survival during systemic viral exposure is a complex task. In the present study, we identified 2-5AS and PKR as IFN-inducible proteins in pancreatic islet cells and linked their induced expression to an enhanced defense against CVB4. Interestingly, our studies also suggested the existence of additional IFN-regulated factors modulating β cell permissiveness to CVB4 in vivo. It is clear that further knowledge about what specific antiviral defense mechanism(s) β cells produce in response to IFNs, as well as on the specific roles of these defense pathways for islet cell survival during CV infection, will lead to possible avenues to modulate the risk for diabetes development following viral infection.

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