



ADENOVIRUS VIRAL ACCESSORY RNAs CAN INDUCE IFN-β EXPRESSION IN L929 CELLS.

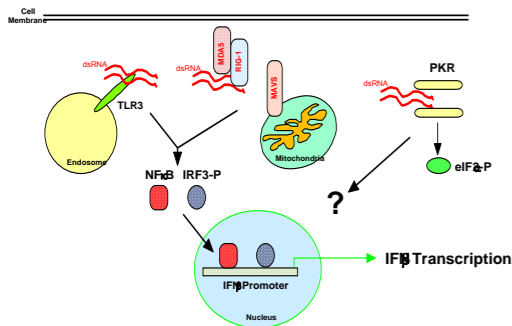
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Abstract

Adenovirus encodes two RNA polymerase III-transcribed small viral associated RNAs (VA_I and VA_{II}) that accumulate to high levels at late stages of infection. Although single-stranded, they undergo intramolecular nucleotide pairings resulting in structures that resemble dsRNA. Previous studies have revealed VA_I enhanced viral replication by blocking protein kinase R (PKR) activity, while the role of VA_{II} has not been fully established. Since VA RNAs are highly structured, we sought to determine whether they can effect interferon-β (IFN-β) expression in mouse cells. Highly purified *in vitro* transcribed RNAs were transfected into L929 cells and the level of IFN-β was determined by ELISA. Both VA RNAs produced a time and dose-dependent increase of IFN-β. Surprisingly, the levels were associated with increased PKR and eukaryotic initiation factor 2 alpha (eIF2α) phosphorylation. Interferon-regulatory factor 3 (IRF3) phosphorylation was not observed suggesting the mechanism(s) of IFN-β expression may be independent of Toll-like receptor 3 (TLR3) and retinoic-acid inducible gene 1 (RIG-I) pathways. The dsRNA polyriboinosinic: polyribocytidylic acid (polyI:C) was studied in parallel and also exhibited increased PKR and eIF2α phosphorylation. In contrast, polyI:C treatment caused increased IRF3 phosphorylation indicating that it can also participate in TLR3 and/or RIG-I signaling. Pre-treatment of L929 cells with the PKR antagonist 2-aminopurine (2-AP) resulted in a dose-dependent inhibition of IFN-β further suggesting PKR played a role in IFN-β expression. Increased IFN-β expression levels were independent of transfection methods and inhibition was not associated with cytotoxicity. Interestingly, VA_{II} RNA consistently induced higher levels of IFN-β as compared to VA_I and polyI:C suggesting this may represent a unique function of this highly structured viral RNA.

Introduction

Signaling pathways involved in dsRNA recognition can lead to the expression of IFN-β. dsRNA activates the innate immune response by interacting with multiple intracellular proteins. Adenovirus type 2 VA RNAs have been previously shown to counteract the cellular antiviral defence mechanism by direct inhibition of PKR thus enhancing its replication. Their potential roles in TLR or RLR signalling have not been fully determined.



Abbreviations: dsRNA, double-stranded RNA; eIF2α, eukaryotic translation initiation factor 2α; IFN-β, interferon β; IRF3, interferon-regulatory factor 3; MAVS, mitochondrial antiviral signalling protein; MDA5, melanoma differentiation-associated gene 5; NF-κB, nuclear factor-kappa B; PKR, dsRNA-activated protein kinase; RIG-I, retinoic-acid inducible gene 1; TLR3, Toll-like receptor 3.



VA I RNA Secondary Structure

Adenovirus VA_I and VA_{II} RNAs

- Highly Expressed (>10⁷ Copies/cell) late in Adenovirus Replication cycle
- VA_I shown to be important for replication (Blocks PKR Activation by dsRNA)
- Role of VA_{II} is unclear
- Can these small structured RNAs affect the innate immune signaling TLR3 and RIG-I pathways (in addition to PKR)?
- Compare VA_I and VA_{II} to poly I:C in transient Transfection assays

Materials and Methods

Reagents

PolyI:C (Midland), 2-AP (Sigma), RNase One Ribonuclease (Promega), Lipofectamine and Lipofectin (Invitrogen).

In vitro transcription of VA_I and VA_{II} RNAs.

Adenovirus type 2 VA_I and VA_{II} genes (pAdVantage vector, Promega) were amplified by PCR with primers containing T7 promoter sequence, then *in vitro* transcribed using MEGashortscript Kit (Ambion), and VA_I and VA_{II} RNAs purified with miRNeasy Mini Kit (Qiagen). Purity and integrity of the purified RNAs was verified by spectrophotometric and electrophoretic methods

Cell Culture and ELISA assay

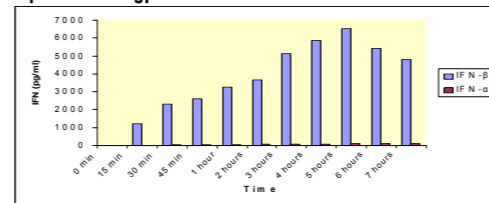
Mouse L929 cells (ATCC, CCL-1) were maintained in Modified Eagle medium (MEM) supplemented with 10% fetal bovine serum (FBS). Before transfection cells were plated at 3x10⁴ cells per well in 96-well plates and incubated overnight to 80% confluency. Transfection with increasing concentrations of VA RNAs or polyI:C was performed using Lipofectamine or Lipofectin, according to the manufacturer's protocol, and cells were incubated at 37°C at 5%CO₂ for the indicated time course. Cell lysates were prepared with Cell Extraction Buffer (BioSource) in the presence of protease inhibitors and tissue culture supernatants collected 18-24 hours post-transfection. The concentration of IFN-α and -β in supernatants was determined by Mouse IFN-α and -β ELISA kits (PBL, 42100-1 and 42400-1, respectively).

Western Blot

5x10⁵ cells were transfected with 0.3μg of polyI:C, VAI or VAII RNAs for different time periods. Cell lysates were analyzed by Western blot using polyclonal primary antibodies against the total [Santa Cruz Biotechnology, M-515] or phosphorylated forms of PKR [Calbiochem, pThr451, 527460]. Primary antibodies against phosphorylated IRF-3 [Ser396, 4961], total or phosphorylated eIF2α [Ser51, 3597] antibodies were purchased from Cell Signalling. Beta Actin Rabbit polyclonal antibody was from EPITOMICS (1784-1). The Anti-Rabbit IgG, HRP-conjugated [Promega, W4011] was used as secondary antibody. The protein bands were visualized by chemiluminescence detection [PerkinElmer, NEL104].

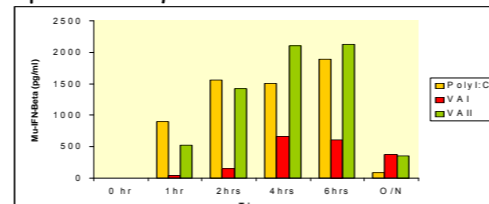
Results

Figure 1. Poly I:C transfection promotes time-dependent expression of type I IFNs.



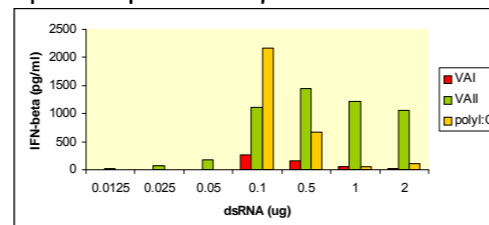
Mouse L929 cells were transfected with 0.1μg of poly I:C. Supernatants were collected at the indicated times and analyzed for IFN-α and IFN-β expression by ELISA.

Figure 2. VA RNA transfection promotes time-dependent expression of IFN-β.



Mouse L929 cells were transfected with 0.1μg of either VA_I or VA_{II} RNAs. Supernatants were collected at the indicated times and analyzed for IFN-β expression by ELISA. Poly I:C was included for comparison.

Figure 3. VA RNA transfection promotes concentration-dependent expression of IFN-β.



Mouse L929 cells were transfected with 0.1μg of either VA_I or VA_{II} RNAs. Supernatants were collected at 24 hours post-transfection and analyzed for IFN-β expression by ELISA. Poly I:C was included for comparison.

Table 1. IFN-β expression is dependent upon intracellular delivery of VA RNAs.

Transfection/cell line	L929	L929 RAW	RAW	RAW
Lipofectamine	no	yes	no	yes
polyI:C	0	668	15	12
VAI RNA	0	102	0	56
VAII RNA	0	1311	0	84

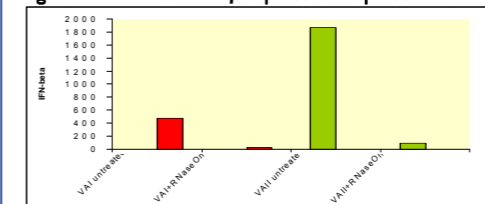
Poly I:C, VAI and VAII RNAs (0.1μg) Mouse L929 or RAW264.7 cells were either added directly to growth media or transfected with Lipofectamine reagent. Supernatants were collected at 24 hours post-transfection and analyzed for IFN-β expression by ELISA (presented in pg/ml).

Table 2. IFN-β expression is observed using different transfection reagents.

dsRNA	Transfection reagent	IFN-β [pg/ml]
VA _I	Lipofectamine	93
VA _{II}	Lipofectamine	1009
PolyI:C	Lipofectamine	491
VA _I	Lipofectin	15
VA _{II}	Lipofectin	564
PolyI:C	Lipofectin	1695

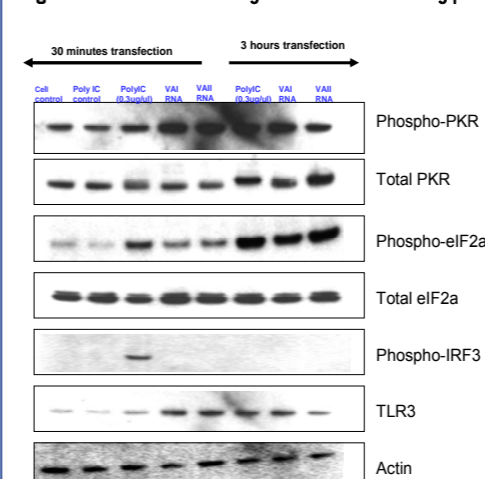
Mouse L929 cells were transfected with 0.1μg polyI:C, VA_I or VA_{II} using either Lipofectamine or Lipofectin as the intracellular delivery agent. Supernatants were collected 18 hours post-transfection and IFN-β levels determined by ELISA.

Figure 4. VA-induced IFN-β expression requires intact RNA.



VA_I and VA_{II} RNAs [10μg] were incubated for 1 hour at 37°C with or without the addition of RNase. Mouse L929 cells were then transfected with 0.1μg treated and mock-treated RNAs. Supernatants were collected at 24 hours post-transfection and analyzed for IFN-β expression by ELISA.

Figure 5. Western Blot analysis of innate immunity proteins.



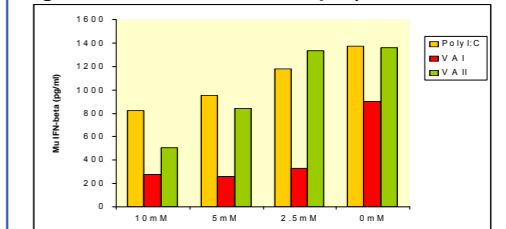
Cell lysates were collected, separated by SDS-PAGE and transferred to nitrocellulose membranes. Subsequently, they were probed with antibodies against the total or phosphorylated forms of PKR, eIF2-α, IRF3 and TLR3. Anti-β-actin antibody was used as an internal protein control.

Table 3. VA_I and VA_{II} RNAs have additive effect on IFN-β expression.

RNA Sample	VAI	VAII	VAI+VAII
Concentration (μg)	0.5	0.1	0.5+0.1
IFN-β [pg/ml]	290	803	1160

Mouse L929 cells were transfected with VA_I and VA_{II} RNAs (alone or in combination). Supernatants were collected at 18 hours post-transfection and analyzed for IFN-β expression by ELISA.

Figure 6. Effects of 2-AP on Mu IFN-β expression.



L929 cells were transfected with polyI:C, VA_I and VA_{II} in the presence of increasing concentrations of the PKR inhibitor 2-AP for 6 hours and tissue culture supernatants collected 18 hours post transfection.

Conclusions

- We have shown that VA_I, VA_{II} RNAs and poly I:C induce the expression level of IFN-β in mouse cells (L929 and RAW cells).
- The increased IFN-β expression levels were independent of the transfection methods used. It showed time- and dose-dependent kinetics.
- We observed >10 fold lower levels of IFN-α produced by mouse cells after transfection with polyI:C, VA_I, and VA_{II} RNAs as compared to the IFN-β levels.
- Our preliminary study suggests that VA_{II} is more potent at inducing IFN-β as compared to VA_I RNA [and polyI:C].
- The levels of IFN-β were associated with increased PKR and eIF2α phosphorylation suggesting that PKR may be involved in the IFN-β production. However, IRF3 phosphorylation was not observed indicating that the mechanism of IFN-β expression may be independent of TLR3 and/or RIG-I pathways.
- Treatment of L929 cells with the PKR antagonist 2-aminopurine (2-AP) at the time of transfection with dsRNA resulted in a dose-dependent inhibition of IFN-β further suggesting PKR played a role in IFN-β expression.
- We are evaluating other kinase inhibitors to determine if PKR phosphorylation is required for VA RNA stimulated IFN-β expression.

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