Adenosine Deaminase Acting on RNA (ADAR1) Suppresses the Induction of Interferon by Measles Virus

Zhiqun Li*, Kristina M. Okonski*, and Charles E. Samuel*

Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, California 93106

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*Contributed equally to this work.

*Corresponding author. Mailing address: Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA 93106.
Phone: (805) 893-3097. Fax: (805) 893-4724. E-mail: samuel@lifesci.ucsb.edu
ABSTRACT

ADAR1, the interferon (IFN) inducible adenosine deaminase acting on RNA, catalyzes the C6 deamination of adenosine (A) to produce inosine (I) in RNA substrates with double-stranded character. Because double-stranded RNA (dsRNA) is a known inducer of IFN, we tested the role of ADAR1 in IFN induction following virus infection. HeLa cells made stably deficient in ADAR1 (ADAR1kd) were compared to vector control (CONkd) and protein kinase PKR-deficient (PKRkd) cells for IFN-β induction following infection with either parental (WT) recombinant Moraten vaccine strain measles virus or isogenic knockout mutants deficient for either V (Vko) or C (Cko) protein expression. We observed potent IFN-β transcript induction in ADAR1kd cells by all three viruses; by contrast, in ADAR1-sufficient CONkd cells only the Cko mutant virus was an effective inducer and the IFN-β RNA induction was amplified by PKR. The enhanced IFN-β transcript inducing capacity of the WT and Vko viruses seen in ADAR1-deficient cells correlated with the enhanced activation of PKR and of interferon regulatory factor IRF3 and activator of transcription ATF2, reaching levels similar to those seen in Cko-infected cells. However, the level of IFN-β protein produced was not proportional to the level of IFN-β RNA, but rather correlated inversely with the level of activated PKR. These results suggest that ADAR1 functions as an important suppressor of MV-mediated responses including activation of PKR and IRF3 and the induction of IFN-β RNA. Our findings further implicate a balanced interplay between PKR and ADAR1 in modulating IFN-β protein production following virus infection.
INTRODUCTION

Measles virus (MV), a member of the Morbillivirus genus of the Paramyxoviridae family, possesses a ~15.9-kb negative-sense single-stranded RNA genome that specifies 6 genes, N, P/V/C, M, F, H, and L, that encode six structural proteins. The P/V/C gene is polycistronic and encodes the V and C nonstructural proteins in addition to the structural protein product, P, an essential cofactor for the viral polymerase (20). Studies of isogenic knockout virus mutants defective for expression of either V (Vko) or C (Cko) have established that these proteins modulate the host response to MV infection (15, 43). The mutants show strong adaptive immune responses, but innate responses are dysregulated (9, 12, 55). Infection with MV causes acute febrile illness. In rare cases, measles infections can progress to a persistent infection of the central nervous system, resulting in the chronic and often fatal disease known as subacute sclerosing panencephalitis (35). Although there is an effective vaccine, measles infections globally continue to cause significant morbidity and mortality. The need for improved MV vaccines and for increased adherence to recommendations with existing vaccines (20), together with the potential for using recombinant MV vaccine strains with defined mechanisms of attenuation as oncolytic therapeutic agents (9), have led to further efforts to better understand the host responses to MV infection at the molecular level.

A cornerstone of the antiviral innate immune response is the interferon (IFN) response. IFN production is triggered by pathogen-associated molecular patterns (PAMPs) that include viral RNAs detected by multiple sensors, among which are the retinoic acid inducible protein (RIG-1)-like cytosolic sensors (RLRs) and the endosomal
membrane-associated Toll-like receptors (TLRs) (24, 60). Measles virus is known to activate the RLR pathway that signals through the mitochondrial adaptor protein IPS-1 to activate IFN-β gene transcription (29, 43) through interferon regulatory factor 3 (IRF3), nuclear factor κB (NFκB) and activating transcription factor 2 (ATF2/c-jun) that form the IFN-β enhanceosome (37).

IFN action involves binding of IFN to cognate receptors and subsequent prototypical JAK-STAT signal transduction leading to transcriptional activation of IFN-stimulated genes, some of which encode protein products that alter virus multiplication (7, 47). Among the IFN-inducible genes are two that encode double-stranded (ds) RNA binding proteins with enzymatic activities: the protein kinase regulated by RNA (PKR); and, the adenosine deaminase acting on RNA (ADAR1). DsRNA is a regulatory effector of PKR, and a substrate of ADAR1 (45, 46, 57). PKR possesses two dsRNA binding domains and a C-terminal catalytic kinase domain (30, 57), and is activated by binding dsRNA or structured ssRNA, leading to autophosphorylation, dimerization and subsequent substrate phosphorylation (4, 5, 28, 32, 45, 57). The best-characterized substrate remains eukaryotic translation initiation factor 2α (eIF2α) (48) in which phosphorylation leads to inactivation, thereby altering the translational pattern in cells and inducing apoptosis. PKR function typically is antiviral and proapoptotic (39, 45, 47).

The adenosine deaminase enzyme ADAR1 catalyzes the C6-deamination of adenosine (A) to inosine (I) in RNA substrates with double-stranded character, a reaction known as A-to-I editing (17, 57). This editing can lead to destabilization of RNA structures, because I:U mismatch pairs are less stable than A:U pairs, or alternatively A-to-I editing can cause changes in genetic decoding during translation or viral RNA...
replication, because I base-pairs as G with C, instead of A with U (3, 46). Among the RNAs selectively edited by ADARs in a manner that affects their translational decoding are cellular pre-mRNAs that code two important types of neurotransmitter receptors (GluR-B, 5HT-2cR) and hepatitis delta virus antigenome RNA (17). Nonselective editing at multiple sites can occur when RNA substrates possess extensive duplex character, as has been described for measles virus and polyoma virus infections (46).

Two size isoforms of ADAR1 protein are generated by a mechanism that involves usage of alternative promoters and alternative splicing (18, 38). A long form of ADAR1 (p150) is IFN inducible and localizes to both the nucleus and the cytoplasm, whereas a short form (p110) is constitutively expressed and localizes predominantly, if not exclusively, to the nucleus (17, 18, 38, 41). Both ADAR1 isoforms, p150 and p110, possess three copies of the dsRNA binding domain first discovered in PKR (27, 38). Remarkably, even though an IFN-stimulated gene, ADAR1 function is often proviral and antiapoptotic or cell protective in nature (16, 39, 46).

Both IFN production and action are impaired in MV-infected cells (43). Antagonism of IFN action occurs through P, V and to some extent also C protein, which inhibits STAT activation and nuclear translocation, thereby impairing induction of ISGs (8, 36, 42, 43, 59). The molecular basis by which IFN production is antagonized is less well understood. The V protein inhibits MDA5-mediated RLR signaling leading to IFN production (1, 10, 12); the C protein also inhibits IFN production (12), possibly indirectly by affecting viral RNA synthesis. Virus expressing both C and V is a poor inducer of IFN, but mutants lacking either V or C are inducers of IFN-β transcription (29). PKR functions to amplify IFN-β RNA induction in an IPS-1-dependent signaling response.
following MV infection (29). The amplification of IFN-β transcript levels by PKR correlates with a PKR-dependent activation of ATF2 and NFκB, whereas the activation of IRF3 following MV infection is PKR-independent (29, 56).

Because ADAR1 has been shown to suppress MV-induced activation of both PKR and IRF3 (56), we hypothesized that ADAR1 might also suppress IFN-β induction by MV. To test the possible role of ADAR1 in IFN-β induction following MV infection, we used HeLa cells stably deficient in ADAR1 (ADAR1\textsuperscript{kd}). Results obtained with wildtype (WT) parental Moraten vaccine strain were compared to those obtained with isogenic V\textsuperscript{ko} and C\textsuperscript{ko} mutants. We found that WT and V\textsuperscript{ko} virus were poor inducers of IFN-β RNA in ADAR1-sufficient cells compared to C\textsuperscript{ko} virus inducing activity as anticipated from prior results (29). However, all three viruses remarkably became robust inducers of IFN-β RNA in ADAR1\textsuperscript{kd} cells. Comparably high levels of IFN-β transcription were found in WT, V\textsuperscript{ko} and C\textsuperscript{ko} infected ADAR1-deficient cells in the presence of PKR, but the level of IFN-β protein produced did not correlate with the steady-state level of IFN-β RNA. Suppression of IFN-β RNA induction in the presence of ADAR1 correlated with suppression of IRF3 phosphorylation and dimerization, and ATF2 phosphorylation, whereas the efficiency of IFN-β protein production correlated inversely with PKR activation. Our results establish ADAR1 as an important modulator of the host interferon response triggered by measles virus infection.
MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% (vol/vol) fetal bovine serum (HyClone), 100 μg/mL of penicillin, and 100 units/ml of streptomycin (GIBCO/Invitrogen) as previously described (63). HeLa cells made stably deficient in either ADAR1 (ADAR1kd) (25, 56) or PKR (PKRkd) (61, 63) by an integrated short hairpin silencing RNA interference strategy utilizing the pSUPER.retro.puro vector were as previously described, as were the drug-resistant knockout vector control cells (CONkd). Knockdown cells were maintained in medium containing 1 µg/mL puromycin (Sigma). The recombinant parental Moraten measles virus vaccine strain, herein designated as WT, includes the gene encoding green fluorescent protein inserted downstream of the viral H gene. The WT virus and isogenic V-deficient (Vko) and C-deficient (Cko) mutants were as described previously (13, 55). Infections were carried out as previously described (29, 55) at a 50 percent tissue culture dose per cell multiplicity of infection (MOI) of 3 unless otherwise noted.

Quantitative real-time PCR. IFN-β and IkBα transcripts were measured by quantitative real-time PCR (qPCR) as previously described (29). Cells seeded in 6-well plates were infected with measles virus, either WT, Vko or Cko, or left uninfected. Total RNA was isolated from cells at 24 h after infection and from uninfected control cells with TRIzol (Invitrogen) according to manufacturer’s instructions. Random-primed reverse transcription was carried out using ~2 µg of RNA and SuperScript II (Invitrogen) according to the manufacturer’s protocol. For PCR analyses, the following primer pairs were used: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward primer GCCTTCCGTGTCCCCACTG and reverse primer CGCCTGCTTCACCACCTTC;
IFN-β forward primer AAACTCATGAGCAGTCTGCA and reverse primer AGGAGATCTTCAGTTTCGGAGG; IκBα forward primer AATTGCTGGGGCAGTCTGGA and reverse primer TAGCCTTCAGGATGGAGTGG.

qPCR reactions were performed in duplicate with each reverse transcription template by using IQ SYBR green Supermix (Bio-Rad) and a Bio-Rad MyIQ real-time qPCR instrument. For IFN-β, the cycle program was: 3-min hot start followed by 30 s at 95°C, 45 s at 58°C, and 45 s at 72°C, repeated 40 times. For IκBα, the cycle program was: 3-min hot start followed by 30 s at 95°C, 30 s at 55°C – 65°C (gradient set as A: 65.0°C B: 64.3°C C: 63.1°C D: 61.2°C E: 58.7°C F: 57.0°C G: 55.8°C H: 55.0°C), and 30 s at 72°C, repeated 40 times. The IFN-β and IκBα values were normalized to GAPDH values.

ELISA for IFN-β. Cells seeded in 6-well plates were infected with measles virus, either WT, Vko or Cko, or left uninfected. Cell culture supernatant fractions (1.5 mL) were harvested 24 h post-infection and assayed for IFN-β protein using a VeriKine Human IFN Beta ELISA kit (Pestka Biomedical Laboratories) as per the manufacturer’s protocol. IFN-β protein levels (pg/mL) were calculated based on a standard curve generated at the time of the assay.

Western immunoblot analysis. Cells were harvested 24 h post-infection and whole cell lysates were prepared as previously described (55). Protein concentration was determined by the Bradford assay method (Bio-Rad). Protein (20-30 μg) was fractionated by either 7% or 10% sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes, and the membranes then were blocked with 5% milk (wt/vol) in phosphate-buffered saline.
For detection of phosphoproteins, membranes were blocked with 5% BSA (wt/vol) and 0.1% Tween-20 (vol/vol) in Tris-buffered saline. Antibodies used to detect specific proteins were as previously described for ADAR1 (38), PKR (Santa Cruz Biotechnology), phospho-Thr446 PKR (Epitomics), IRF3 (Santa Cruz Biotechnology), phospho-Ser396 IRF3 (Cell Signaling), ATF2 (Santa Cruz Biotechnology), phospho-Thr71 ATF2 (Cell Signaling), GFP (Santa Cruz Biotechnology), β-actin (Sigma) and α-tubulin (Sigma). Antibody against MV H protein was generously provided by R. Cattaneo (Mayo Clinic, Rochester, MN) as previously described (55). Detection of immunoblots was performed as previously described (29) using the Odyssey infrared imaging system (Li-COR).

**IRF3 dimerization assay.** IRF3 dimer formation was measured by native PAGE essentially as described by Sankar et al. (49). Extracts were prepared from cells, either infected or uninfected, using extract buffer (20 mM HEPES, pH7.9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% NP-40, 1X complete protease inhibitor cocktail (Sigma) and 1X phosphatase inhibitor cocktail (Sigma). Protein fractionation was on 8% acrylamide gels using 25 mM Tris pH 8.3; 192 mM glycine running buffer in the anode chamber, and running buffer supplemented with 1% sodium deoxycholate (DOC) in the cathode chamber. Gels were pre-run at 40 mA for 30 min prior to loading samples; electrophoresis was at 20 mA until the dye reached near the bottom of the gel. Proteins were transferred to a nitrocellulose membrane using ice-cold Tris-glycine buffer at 150 V for 90 min; monomer and dimer IRF3 detection was then by western immunoblot assay with IRF3 antibody or phospho-Ser396 IRF3.
RESULTS

ADAR1 suppresses PKR activation and the induction of interferon beta RNA by measles virus. To test the effect of ADAR1 compared to PKR on IFN-β induction by MV, we utilized HeLa cell clonal lines in which either ADAR1 or PKR was stably knocked down by an shRNA interference strategy. We used the recombinant Moraten vaccine strain of measles virus because entry by MV is through the CD46 receptor that is present on HeLa cells, and because isogenic mutants of the parental (WT) Moraten virus lacking either V or C protein expression (V\(^{ko}\), C\(^{ko}\)) are available (33, 55). The ADAR1\(^{kd}\) cells have less than 15% of the ADAR1 p110 and p150 proteins found in drug-treated CON\(^{kd}\) or PKR\(^{kd}\) cells, and the PKR expression level in PKR\(^{kd}\) cells is less than 5% of PKR-sufficient CON\(^{kd}\) or ADAR1\(^{kd}\) cells (Fig. 1A) as previously described (25, 56, 61).

In ADAR1 sufficient CON\(^{kd}\) cells only the C\(^{ko}\) mutant efficiently activated PKR as measured by phosphorylation of Thr446 (Fig. 1A, lane 4); the level of phospho-PKR in WT and V\(^{ko}\) infected cells remained low and similar to uninfected (UI) cells (Fig. 1A,B). By contrast, in the ADAR1-deficient (ADAR1\(^{kd}\)) cells (Fig. 1A, lanes 9-12), both WT and V\(^{ko}\) virus infections increased PKR Thr446 phosphorylation to a comparably high level as that seen in C\(^{ko}\)-infected cells (Fig. 1B). The PKR\(^{kd}\) cells did not show detectable virus-mediated phosphorylation due to the low level of PKR present in the cells (Fig. 1A, lanes 5-8). Growth of MV was assessed by measuring the viral H and GFP protein expression. Consistent with earlier findings (55, 56), both H and GFP were reduced in ADAR1\(^{kd}\) cells, most notably in the C\(^{ko}\)-infected cells, whereas C\(^{ko}\)-infected PKR\(^{kd}\) cells showed increased protein expression reaching levels comparable to that observed with WT and V\(^{ko}\) viruses (Fig. 1A).
Because we had observed that PKR acts to amplify induction of IFN-β via IPS-1 dependent signaling following either infection with V^{ko} or C^{ko} MV or transfection with dsRNA (28, 29), and because ADAR1 deficiency results in substantially enhanced activation of PKR following MV infection as illustrated by the results shown in Figure 1A, we tested the effect of ADAR1 deficiency on the induction of IFN-β transcripts by MV. The results of a representative real-time quantitative PCR data set are shown in Figure 2. Consistent with prior observations (29), WT and V^{ko} mutant MV were both poor inducers of IFN-β RNA compared to C^{ko} virus in CON\textsuperscript{kd} (ADAR-sufficient, PKR-sufficient) cells; the induction by C^{ko} virus was amplified by PKR, as the IFN-β transcript level in CON\textsuperscript{kd} cells was typically 3- to 5-fold greater in the CON\textsuperscript{kd} cells than in PKR\textsuperscript{kd} cells with the C^{ko} mutant (Fig. 2). In striking contrast to ADAR1-sufficient cells, in ADAR1-deficient cells, the inducing capacity of the WT and V^{ko} viruses was not severely impaired. WT and V^{ko} viruses were both potent inducers of IFN-β RNA in ADAR1\textsuperscript{kd} cells, with the induction increased by ~100-fold in the ADAR1\textsuperscript{kd} cells compared to either CON\textsuperscript{kd} or PKR\textsuperscript{kd} cells. The induction of IFN-β transcripts in ADAR1\textsuperscript{kd} cells by WT and V^{ko} viruses was comparable to that seen with the C^{ko} virus (Fig. 2). Analysis of IFN-β transcript levels in three independent experiments by the Student’s t-test gave $p > 0.7$, for C^{ko}-infected ADAR1\textsuperscript{kd} cells versus WT or V^{ko} infected ADAR1\textsuperscript{kd} cells. By contrast, comparison of IFN-β transcript levels in WT and V^{ko} infected CON\textsuperscript{kd} (or PKR\textsuperscript{kd}) versus WT and V^{ko} infected ADAR1\textsuperscript{kd} cells gave $p < 0.0001$.

IRF3 activation is enhanced in ADAR1-deficient cells following measles virus infection. Because virus-induced activation of IRF3 is an important contributor to the transactivation of IFN-β expression, including in MV-infected cells through IPS-1
dependent signaling (26, 29, 37), we next examined IRF3 activation levels in ADAR1\textsuperscript{kd} cells infected with WT, V\textsuperscript{ko} and C\textsuperscript{ko} viruses compared to CON\textsuperscript{kd} or PKR\textsuperscript{kd} infected cells or uninfected cells as controls (Fig. 3). Activation was assessed by measuring both virus-induced phosphorylation of IRF3 at Ser396 (Fig. 3A,B) and virus-induced IRF3 dimerization (Fig. 3C,D). While C\textsuperscript{ko} virus induced Ser396 phosphorylation in both CON\textsuperscript{kd} and PKR\textsuperscript{kd} cells as measured by western analysis, only low levels of IRF3 phosphorylation were seen in these cells infected with either WT or V\textsuperscript{ko} virus (Fig. 3A,B). By contrast, in the ADAR1\textsuperscript{kd} cells, comparably high levels of phospho-IRF3 were seen following infection with WT and V\textsuperscript{ko} viruses as with C\textsuperscript{ko} virus (Fig. 3A,B). Similar results were seen when IRF3 dimerization was measured by polyacrylamide gel electrophoresis using native gels. C\textsuperscript{ko} virus induced a comparably high level of IRF3 dimerization in all three types of cells, CON\textsuperscript{kd}, PKR\textsuperscript{kd} and ADAR1\textsuperscript{kd}, whereas the WT and V\textsuperscript{ko} viruses induced efficient IRF3 dimerization only in the ADAR1 deficient ADAR1\textsuperscript{kd} cells, not in the CON\textsuperscript{kd} or PKR\textsuperscript{kd} cells (Fig. 3C,D). The slightly reduced mobility of total IRF3 on SDS-PAGE seen in C\textsuperscript{ko}-infected cells correlated with C-terminal Ser396 phosphorylation (Fig. 3A) as previously described (26, 29, 61). IRF3 protein levels were comparable in uninfected CON\textsuperscript{kd}, PKR\textsuperscript{kd} or ADAR1\textsuperscript{kd} cells, and neither phosphorylation at Ser396 (Fig. 3A) nor dimerization (Fig. 3C) of IRF3 was detected to any significant extent in uninfected cells. Finally, the finding that the IRF3 phosphorylation at Ser396 (Fig. 3A,B) and the extent of IRF3 dimerization (Fig. 3C,D) in C\textsuperscript{ko}-infected CON\textsuperscript{kd} and PKR\textsuperscript{kd} cells are comparable suggests that IRF3 phosphorylation is PKR independent. The enhanced activation of
IRF3 seen in ADAR1 deficient cells (Fig. 3) is consistent with the enhanced induction of IFN-β seen in the ADAR1\textsuperscript{kd} cells (Fig. 2).

**ATF2 phosphorylation is enhanced in ADAR1-deficient cells following infection with WT and V\textsuperscript{ko} measles virus.** In ADAR1-sufficient and -deficient cells, the different levels of IFN-β induction by the C\textsuperscript{ko} virus correlated with the phosphorylation of PKR on Thr446 as shown in Figures 1 and 2. Because activation of MAP kinase signaling and ATF2 phosphorylation shows PKR dependency (29, 62), and because PKR activation is enhanced in ADAR1-deficient cells (Fig. 1), we examined the effect of ADAR1 deficiency on the phosphorylation level of ATF2, directly by western immunoblot analysis with monoclonal antibody specific for ATF2 phospho-Thr71 and indirectly by reduction of ATF2 protein mobility on SDS-PAGE (Fig. 4).

The phosphorylation of ATF2 was increased in the CON\textsuperscript{kd} and PKR\textsuperscript{kd} cells, both ADAR1-sufficient, following infection with C\textsuperscript{ko} virus but not with either WT or V\textsuperscript{ko} virus (Fig. 4). However, in ADAR1-deficient cells, infection with all three viruses (WT, V\textsuperscript{ko}, C\textsuperscript{ko}) gave rise to reduced gel mobility of the ATF2 protein and increased phospho-Thr71 levels compared to uninfected cells (Fig. 4). Uninfected CON\textsuperscript{kd}, PKR\textsuperscript{kd} and ADAR1\textsuperscript{kd} cells show similar levels of total ATF2 protein.

**Induction of IκBα is increased in ADAR1-deficient compared to –sufficient cells.** Among the transcripts dependent upon NFκB for transcriptional activation, in addition to IFN-β, is IκBα (22, 37). As a beacon for activation of NFκB (54), we measured IκBα transcript levels by real-time qPCR in ADAR1-sufficient compared to ADAR1-deficient cells following MV infection (Fig. 5). When ADAR1\textsuperscript{kd} cells were infected with either WT or the V\textsuperscript{ko} mutant, a ~10- to 20-fold higher level of IκBα RNA was observed.
compared to the low level seen in ADAR1-sufficient (CON\textsuperscript{kd} or PKR\textsuperscript{kd}) cells infected with either WT or V\textsuperscript{ko} virus or uninfected cells. However, infection with MV C\textsuperscript{ko} mutant virus showed similarly high I\kappaB\alpha transcript levels in ADAR1-sufficient (CON\textsuperscript{kd}) and ADAR1-deficient (ADAR1\textsuperscript{kd}) cells both of which are PKR-sufficient, whereas the transcript level was reduced in PKR-deficient, ADAR1-sufficient (PKR\textsuperscript{kd}) cells (Fig. 5).

**ADAR1 deficiency leads to decreased efficiency of IFN-β protein production.** Because in ADAR1-deficient cells the level of IFN-β transcript is increased following measles virus infection (Fig. 2), but the activation of PKR is also increased in the ADAR1\textsuperscript{kd} cells (Fig. 1B), and because C\textsuperscript{ko} virus infection gave rise to both increased IFN-β RNA transcript and increased PKR activation in the ADAR1\textsuperscript{kd} cells (Figs. 1 and 2), we tested whether IFN-β protein was differentially produced and secreted in the infected cells in an ADAR1-dependent manner. We performed ELISA analyses, and as shown in Table 1, the C\textsuperscript{ko} virus was a better inducer of IFN-β protein than the WT and V\textsuperscript{ko} viruses as anticipated for all three cells, CON\textsuperscript{kd}, PKR\textsuperscript{kd} and ADAR1\textsuperscript{kd}. However, the level of the IFN-β protein was only about 2-fold higher in the C\textsuperscript{ko}-infected compared to WT or V\textsuperscript{ko}-infected PKR-sufficient (CON\textsuperscript{kd}, ADAR1\textsuperscript{kd}) cells, and about 7-fold higher in the PKR-deficient cells. While the C\textsuperscript{ko} virus was the best inducer of IFN-β protein as expected, unexpectedly the level of IFN-β protein produced was less than anticipated from the level of IFN-β RNA transcript (Table 2). When the relative amount of IFN-β protein produced per amount of IFN-β RNA expressed was considered, as an index of the relative efficiency of IFN-β production, the efficiency was highest in the PKR-deficient cells and lowest in the ADAR1-deficient cells that display high levels of PKR activation.
The relative efficiencies in the PKR$_{kd}$ cells (8.63, 3.92, 7.85) were about 10 to 30-fold higher than in the ADAR1$_{kd}$ cells (0.27, 0.34, 0.57) for all three viruses (Table 2).

**DISCUSSION**

The objective of our study was to test whether ADAR1 plays a role in virus-induced IFN-β expression using measles virus, and whether any of the observed ADAR1-dependent responses were modulated by the viral V or C accessory proteins. We found, using cells stably deficient in ADAR1, that IFN-β RNA expression was increased ~100-fold or more following virus infection. Most strikingly, WT and mutant viruses defective for either V or C protein expression all induced a comparably high level of IFN-β RNA in ADAR1-deficient cells, but in ADAR1-sufficient cells only the C$^{ko}$ mutant was a strong inducer. ADAR1 deficiency was accompanied by enhanced activation of signaling factors known to amplify IFN-β RNA expression including IRF3, ATF2 and PKR. While the C$^{ko}$ mutant also induced higher IFN-β protein levels than either WT or V$^{ko}$ virus, the efficiency of production of IFN-β protein was markedly decreased in the ADAR1-deficient cells that showed enhanced activation of PKR compared to the ADAR1-sufficient cells.

Our studies were undertaken because of the following reasons: ADAR1 is known to destabilize dsRNA structure and dsRNA is a potent inducer of IFN and activator of PKR (46, 47); PKR amplifies IFN-β transcript production in ADAR1-sufficient cells infected with either V$^{ko}$ or C$^{ko}$ mutant virus but not WT virus (29); and, enhanced
activation of PKR is observed in ADAR1-deficient HeLa cells (25, 55). Therefore, we anticipated that if PKR activation was central to amplification of IFN-β RNA expression, and if ADAR1 deficiency leads to an increased virus-induced activation of PKR, then ADAR1-deficiency might result in increased expression of IFN-β transcripts. This indeed was observed. But unexpectedly, all three viruses, WT as well as the isogenic mutants defective for either V or C protein expression, were robust inducers of IFN-β RNA in ADAR1-deficient cells, whereas in ADAR1-sufficient cells only the C mutant was a robust inducer. Furthermore, the efficiency of production of the IFN-β protein per steady state amount of IFN-β RNA was substantially reduced in the ADAR1-deficient compared to -sufficient cells in a manner that correlated with increased PKR activation, indicating a balanced interplay between ADAR1 and PKR in modulating IFN-β production following infection.

Induction of the IFN-β gene transcription by virus involves activation of IRF3 and NFκB by RLR or TLR signaling and these factors function together with ATF2/c-jun to constitute the IFN-β enhanceosome that drives IFN-β transcription (37, 43). In the case of MV Moraten infection via CD46, as occurs in HeLa cells (33, 55), activation of IRF3, NFκB and ATF2 are maximal in the absence of the C protein and are IPS-1-dependent, whereas in the absence of the V protein or with WT virus, activation is low and IFN-β induction is poor (29). Our results are both consistent with, and extend, these observations. We found that IRF3 activation was maximal in C$^{ko}$-infected cells, but that the IRF3 activation was PKR-independent. We also found that IRF3 activation was low in WT and V$^{ko}$ infected ADAR1-sufficient cells as earlier reported (29), but in ADAR1$^{kd}$ cells the activation of IRF3 and ATF2 was robust and similarly high to that seen for C$^{ko}$.
virus (Fig. 3, 4). The same was observed when IκBα transcript levels were measured as a beacon of NFκB activity, raising the interesting possibility that NFκB-dependent expression of cytokines in addition to IFN-β may be enhanced in ADAR1-deficient cells following infection. The IκBα RNA expression pattern (Fig. 4) was similar to that for IFN-β RNA expression (Fig. 2), where WT and Vko viruses induced IFN-β as robustly as the Cko virus did in the ADAR1kd cells, but induction was not seen in either of the ADAR1 sufficient cell lines (CONkd, PKRkd). For ATF2 and NFκB activation, our results are in agreement with earlier observations that found that virus induced activation was enhanced by PKR (29, 62). The fact that IFN-β RNA induction and the activation of PKR, IRF3 and ATF2 all were enhanced in the ADAR1 deficient MV-infected cells suggests the accumulation of RNA structures that trigger IFN induction signaling at an upstream point, although the existence of different RNA structures that trigger the activation of different signaling proteins is an alternative possibility. Our results that the knockdown of ADAR1 in HeLa cells reveals enhanced IFN-β transcript expression are consistent with a recent mouse knockout study that also implicates ADAR1 as a suppressor of the IFN response. Genetic disruption of ADAR1 causes embryonic lethality in mice (21, 58) and results in the up-regulation of ISG expression in hematopoietic stem cells in the absence of infection (21).

Dimerization of IRF3 was typically detectable at a low level in WT and Vko virus infected cells under conditions where Ser396 phosphorylation of IRF3 was not readily increased. This disparity may reflect a different sensitivity between the western immunoblotting and native gel electrophoresis assays, or alternatively and perhaps more likely, that phosphorylation at a site other than Ser396 may also contribute to
dimerization of IRF3. The phosphorylation of IRF3 has been described to occur at multiple sites in a two-step process, initially at C-terminal serine/threonine sites between amino acids 396 and 405 and then at Ser385 and Ser386 (23, 37, 61).

Among our most striking results are that in addition to the fact that ADAR1 deficiency led to increased IFN-β transcript levels particularly in WT and V^{ko} infected ADAR1^{kd} cells compared to ADAR1-sufficient cells (Fig. 1) and also increased IFN-β protein (Table 1), the efficiency of IFN-β protein production was notably reduced in the ADAR1^{kd} cells (Table 2). What, then, is the relationship of ADAR1 as an RNA sensor that destabilizes RNA structures (3, 46) relative to PKR as an established regulator of translation (45, 47, 48)?

The efficient IFN-β RNA induction seen in ADAR1^{kd} cells (Fig. 2) was accompanied by an increased activation of PKR (Fig. 1), a known inhibitor of translation (45, 48). Decreased MV replication and increased virus-induced apoptosis also has been described for the ADAR1^{kd} cells (55, 56). We assessed the growth of WT, C^{ko} and V^{ko} viruses by measurement of H and GFP proteins in ADAR1^{kd}, PKR^{kd} and CON^{kd} cells and the results (Fig. 1A) were in good agreement with the published studies (55, 56). While viral protein expression was reduced most extensively in C^{ko} as well as V^{ko}-infected ADAR1^{kd} cells, the extent of the reduction depended upon the protein examined, with the reduction of H generally more pronounced than that of GFP. The inhibition seen with WT virus was more variable, possibly suggesting that PKR activation is not the sole RNA-dependent response modulated by ADAR1 that contributes to the observed decrease in viral gene expression. Replication of WT, V^{ko} and C^{ko} viruses was reduced in the ADAR1^{kd} cells compared to ADAR1 sufficient cells as reported (56), whereas
IFN-β RNA induction was greatly enhanced in the ADAR1<sup>kd</sup> cells as shown herein (Fig. 4). While WT and V<sup>ko</sup> viruses replicate efficiently in cells sufficient in both ADAR1 and PKR, the C<sup>ko</sup> mutant displays a growth restriction phenotype that is partially rescued in PKR<sup>kd</sup> cells (55) but not ADAR1<sup>kd</sup> cells (56). WT and V<sup>ko</sup> viruses were both poor inducers of IFN-β transcripts in the ADAR1-sufficient, PKR-sufficient cells where they replicate to high levels, but C<sup>ko</sup> virus that replicates relatively poorly was an efficient IFN-β RNA inducer. Our findings that PKR as well as ADAR1 contributes to IFN-β transcriptional induction in a pronounced manner, particularly with the C<sup>ko</sup> measles virus mutant, are consistent with recent observations with human parainfluenza virus type 1 C mutants that produced increased IFN-β and increased dsRNA that activated MDA5 and PKR (6). However, our finding of the enhanced activation of PKR in C<sup>ko</sup>-infected control cells, and importantly also in ADAR1<sup>kd</sup> cells infected with WT or V or C mutant virus, is consistent with translational control as a likely explanation for the decreased efficiency of IFN-β protein production seen in these cells (Table 2). Whether translational control mediated by PKR also accounts for the enhanced IFN-β transcript levels seen in the cells is yet to be established.

The MV V protein inhibits RLR signaling likely directly, and the C protein potentially indirectly by modulating viral RNA synthesis (1, 2, 10, 31, 44, 51). Our results are consistent with the notion that in the absence of C protein, aberrant viral RNAs with sufficient structure to activate PKR and RLR signaling are produced, and that even in the presence of ADAR1 which would destabilize dsRNA (3, 46), viral dsRNA of sufficient concentration and structure accumulates to trigger dsRNA sensors including PKR and IPS-1-mediated RLR signaling. According to this possible explanation, in the
presence of C protein, as would be the case with WT and V<sup>ko</sup> viruses, the level of activating RNAs is low because of two reasons: reduced activating RNA synthesis; and, decreased accumulation because of the action of ADAR1. Whereas, in the absence of ADAR1, as in the ADAR1<sup>kd</sup> cells, the concentration of activator dsRNA is sufficiently high to trigger the signaling pathways. The extent to which viral dsRNA structural features overlap for recognition by ADAR1, PKR and the RLRs as foreign dsRNA sensors is presently unknown. This question is presently under investigation by others and us. Length of dsRNA and presence of 5'<sup>-</sup>-triphosphate on ssRNA are among the parameters important for IPS-1-dependent RLR signaling and PKR activation (32, 45, 60). The potential importance of the differential activation of dsRNA sensors, for example IPS-1-dependent signaling involved in transcriptional induction of IFN-β gene expression (60) and PKR that down-regulates translation (45, 47), is illustrated by the increased expression of IFN-β transcripts but decreased efficiency of IFN-β protein production observed in the ADAR1-deficient, PKR-sufficient cells (Table 2).

PKR is a well-established ISG of central importance in the antiviral actions of IFNs (47). More recent evidence implicates PKR as a key player in the induction of IFN-β, not only by MV (29) but also by other RNA viruses (6, 19, 52, 53). By contrast, we found that ADAR1, also an ISG, displayed proviral activity with MV in HeLa cells (56). Similarly, with VSV, ADAR1 was found to be proviral both in MEFs and HeLa cells by suppressing the activation of PKR phosphorylation (25, 34). Several independent reports concluded that ADAR1 was proviral and increased HIV replication (11, 14, 40). Furthermore, in a screen of ~380 ISGs by overexpression, ADAR1 was identified as one of a few ISGs that significantly enhanced the replication of multiple viruses including...
HIV, West Nile virus, Venezuelan equine encephalitis virus and yellow fever virus (50).

While antagonism of PKR activation is emerging as one mechanism by which ADAR1 may enhance virus replication (16, 46), another potentially more broadly operative mechanism relates to the suppression of virus-induced production of IFN-β RNA in ADAR1-sufficient cells as observed herein (Fig. 2).
This work was supported in part by research grants AI-12520 and AI-20611 to CES from the National Institute of Allergy and Infectious Diseases, NIH, U.S. Public Health Service, and by a postdoctoral fellowship award to ZL from the Santa Barbara Foundation.
FIGURE 1. ADAR1 deficiency enhances PKR activation following infection with measles WT, V<sup>ko</sup> and C<sup>ko</sup> viruses. (A) CON<sup>kd</sup>, PKR<sup>kd</sup> and ADAR1<sup>kd</sup> cells were either left uninfected (UI) or infected with WT, V<sup>ko</sup> or C<sup>ko</sup> measles virus as indicated. At 24 h post infection whole cell extracts were prepared and analyzed by western immunoblot assay with antibodies against ADAR1, PKR, phospho-Thr446-PKR, MV H, GFP and α-tubulin. XRP marks position of cross-reacting protein with ADAR1 antibody (slower mobility than p150); arrowhead marks postulated caspase-mediated p120 ADAR1 cleavage product. (B) Quantification of fold activation of PKR, as measured by the level of phospho-Thr446-PKR to total PKR, determined by western immunoblot analysis as shown in (A). * , p < 0.05 by Student’s t-test for comparison of PKR activation in uninfected cells versus cells infected with WT, V<sup>ko</sup> or C<sup>ko</sup> measles viruses. The results shown are the means with S.E. values from three independent experiments.

FIGURE 2. ADAR1 deficiency results in enhanced induction of IFN-β transcripts following infection with measles virus. CON<sup>kd</sup>, PKR<sup>kd</sup> and ADAR1<sup>kd</sup> cells were left uninfected (UI) or were infected with WT or mutant (V<sup>ko</sup> or C<sup>ko</sup>) MV as indicated. Total RNA was isolated at 24 h after infection and IFN-β mRNA levels normalized to GAPDH were determined using quantitative real-time PCR.

FIGURE 3. IRF3 activation is enhanced in ADAR1 deficient cells following infection with measles WT, V<sup>ko</sup> and C<sup>ko</sup> viruses. CON<sup>kd</sup>, PKR<sup>kd</sup> and ADAR1<sup>kd</sup> cells were either left uninfected (UI) or infected with WT, V<sup>ko</sup> or C<sup>ko</sup> measles virus as indicated. (A) At 24 h post infection whole cell extracts were prepared and analyzed by western immunoblot assay with antibodies against IRF3, phospho-Ser396-IRF3, and α-
tubulin. (B) Quantification by western immunoblot analysis of the fold activation of IRF3 as measured by the level of phospho-Ser396-IRF3 to total IRF3. * p < 0.05 by Student’s t-test for comparison of IRF3 activation in uninfected cells versus cells infected with WT, V^ko and C^ko viruses. The results shown are means with S.E. values (n=3). (C) IRF3 activation as measured by dimer formation. Extracts were prepared and analyzed for IRF3 dimer formation by native polyacrylamide gel electrophoresis as described under the Material and Methods. The blot was probed with antibody against IRF3 to detect dimer complexes fractionated from monomer protein. (D) Quantification of dimerization as measured by the amount of IRF3 dimer to total IRF3 (monomer + dimer). The results shown are means with S.D. values (n=3).

FIGURE 4. Effect of deficiency of ADAR1 or PKR on measles virus-induced activation of ATF2. CON^kd, PKR^kd and ADAR1^kd cells were either left uninfected (UI) or infected with WT, V^ko or C^ko measles virus as indicated. At 24 h post infection whole cell extracts were prepared and analyzed by western immunoblot with antibodies against ATF2, phospho-Thr71-ATF2, and α-tubulin.

FIGURE 5. ADAR1 deficiency results in enhanced induction of IκBα following measles virus infection. CON^kd, PKR^kd and ADAR1^kd cells were either left uninfected (UI) or infected with WT, V^ko or C^ko measles virus as indicated. At 24 h post infection total RNA was isolated and IκBα mRNA normalized to GAPDH was measured by quantitative real-time PCR.
REFERENCES


TABLE 1
Effect of deficiency of ADAR1 or PKR on expression of IFN-β protein production in response to measles virus infection

<table>
<thead>
<tr>
<th>Cell</th>
<th>Virus</th>
<th>IFN-β (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON kd</td>
<td>UI</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>464 ± 23</td>
</tr>
<tr>
<td></td>
<td>Vko</td>
<td>406 ± 46</td>
</tr>
<tr>
<td></td>
<td>Cko</td>
<td>985 ± 68</td>
</tr>
<tr>
<td>PKR kd</td>
<td>UI</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>354 ± 21</td>
</tr>
<tr>
<td></td>
<td>Vko</td>
<td>310 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>Cko</td>
<td>2558 ± 371</td>
</tr>
<tr>
<td>ADAR1 kd</td>
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<td>&lt;10</td>
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<tr>
<td></td>
<td>WT</td>
<td>533 ± 39</td>
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<tr>
<td></td>
<td>Vko</td>
<td>743 ± 66</td>
</tr>
<tr>
<td></td>
<td>Cko</td>
<td>1178 ± 32</td>
</tr>
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</table>

CONkd, PKRkd or ADAR1kd cells in 6-well plates were infected with either WT, Vko or Cko virus or left uninfected. IFN-β accumulation in cell culture supernatant fractions (1.5 mL) after 24 h was measured by ELISA as described in Materials and Methods. Results are means with S.E. values (n=4).
<table>
<thead>
<tr>
<th>Cell</th>
<th>Virus</th>
<th>IFN-β RNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IFN-β protein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Expression Efficiency (relative)</th>
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<tr>
<td>CON&lt;sup&gt;kd&lt;/sup&gt;</td>
<td>WT</td>
<td>82</td>
<td>464</td>
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<tr>
<td></td>
<td>V&lt;sup&gt;ko&lt;/sup&gt;</td>
<td>147</td>
<td>406</td>
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<tr>
<td>PKR&lt;sup&gt;kd&lt;/sup&gt;</td>
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<td>0.57</td>
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The relative expression efficiency was calculated from the amount of IFN-β protein per amount of IFN-β RNA expressed. Relative expression efficiency = (IFN-β protein)/(IFN-β RNA). <sup>a</sup> IFN-β RNA transcript level determined by qPCR from Figure 2. <sup>b</sup> IFN-β protein produced determined by ELISA from Table 1.
Figure 1A
Figure 1B
Figure 2

IFN-β induction (fold)

CONkd

PKRkd

ADAR1kd

UI WT Vko Cko

UI WT Vko Cko

UI WT Vko Cko

UI WT Vko Cko

Figure 2
Figure 3A
Figure 3B
Figure 3C
Figure 3D
Figure 4
Figure 5