Stress Granule Formation Induced by Measles Virus is Protein Kinase PKR-dependent and Impaired by RNA Adenosine Deaminase ADAR1

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ADAR1, an interferon (IFN) inducible double-stranded (ds) RNA-specific adenosine deaminase, down regulates host innate responses including activation of the dsRNA-dependent protein kinase (PKR) and induction of IFN-β mRNA. Conversely, PKR amplifies IFN-β induction by measles virus (MV) and inhibits virus protein synthesis. Formation of stress granules (SGs), cytoplasmic aggregates of stalled translation complexes and RNA binding proteins, are a host response to virus infection mediated by translation initiation factor eIF2α. We examined the roles of PKR and ADAR1 in SG formation using HeLa cells stably deficient in either PKR (PKR<sup>kd</sup>) or ADAR1 (ADAR1<sup>kd</sup>) compared to control (CON<sup>kd</sup>) cells. Infection with either wildtype (WT) MV or an isogenic mutant lacking C protein expression (C<sup>ko</sup>) comparably induced formation of SG in ADAR1<sup>kd</sup> cells, whereas only the C<sup>ko</sup> mutant was an efficient inducer in control cells. Both ADAR1 and PKR colocalized with SG following infection. MV-induced SG formation was PKR-dependent but impaired by ADAR1. Complementation of ADAR1<sup>kd</sup> cells by expression of either p150 WT isoform or the p150 Zα (Y177A) Z-DNA binding mutant of ADAR1 restored suppression of host responses including SG formation and PKR activation. By contrast, neither the p110 WT isoform nor the p150 catalytic (H910A,E912A) mutant of ADAR1 complemented the ADAR1<sup>kd</sup> phenotype. These results further establish ADAR1 as a suppressor of host innate responses including activation of PKR and the subsequent SG response.
Measles virus (MV) possesses a negative-sense single-stranded RNA genome of ~15.9-45 kb and is member of the *Morbillivirus* genus of the *Paramyxoviridae* family. From the six MV specified genes, N, P/V/C, M, F, H and L, eight proteins are encoded: five structural proteins from monocistronic genes, whereas the polycistronic P/V/C gene encodes the structural P protein, a polymerase cofactor, in addition to the V and C accessory proteins that suppress the host immune response (19). Infection with MV causes acute febrile illness, and despite the existence of an effective vaccine, MV remains a leading cause of morbidity and mortality worldwide (24). Persistent infection with MV, although rare, may lead to a serious and often fatal neurodegenerative disease, subacute sclerosing panencephalitis (45). The potential for use of attenuated MV strains as oncolytic agents for cancer therapy (14), coupled with the need for improved MV vaccines as well as increased adherence to vaccine regimens (19), have motivated research to further understand the molecular mechanisms of the host response to MV infection. MV is immunomodulatory, with the capacity to affect both the innate and adaptive immune responses in part through the functions of the V and C accessory proteins as revealed from studies using isogenic knockout mutant viruses defective for either V (Vko) or C (Cko) protein expression (49).

The interferon (IFN) system plays a central role in the innate antiviral immune response to virus infection. Pathogen-associated molecular patterns of viruses, including structural characteristics of viral RNAs that differentiate them from cellular RNAs, trigger the transcriptional activation of IFN through multiple signaling pathways. Among these are the cytoplasmic retinoic-acid-inducible protein (RIG-I) sensors that detect viral
RNAs including MV RNA as foreign RNA (40, 49, 73). IFNs are produced and secreted and can act in autocrine and paracrine manners. Following binding to their cognate receptors, IFNs trigger signaling including by the canonical JAK-STAT pathway that leads to the induction of several interferon-stimulated genes (ISGs), some of which encode proteins with antiviral activities (52). Among the ISGs are two enzymes that are dsRNA-binding proteins, the protein kinase PKR regulated by dsRNA and the adenosine deaminase ADAR1 that acts on dsRNA (46, 47, 49, 51, 56).

The PKR kinase is an IFN-inducible kinase that is either activated or antagonized following binding of dsRNA or single-stranded RNA (ssRNA) with ds character. PKR typically displays proapoptotic and antiviral activities (47, 51, 52, 66). DsRNA bound to a repeated domain present in the N-terminal region of PKR can lead to activation by autophosphorylation including at threonine 446, dimerization, and subsequent substrate phosphorylation catalyzed by the kinase subdomains present in the C-terminal region of the PKR protein (41, 66). The best-characterized PKR substrate, eIF2α (53), when phosphorylated alters the translational pattern in cells which can lead to apoptosis (51, 52).

The ADAR1 RNA deaminase, like PKR, is an IFN-inducible enzyme, but in the case of ADAR1, dsRNA is typically the substrate rather than an effector of activity as in the case of PKR (56, 66). ADAR1 catalyzes the C-6 deamination of adenosine (A)-to-inosine (I) in dsRNA, a process known as A-to-I editing (21, 66). ADAR1 editing activity is a physiologically relevant process that can cause changes in genetic decoding at the translational level and also lead to destabilization of RNA structures (9, 56). Dependent upon their structure, substrate RNAs are either selectively edited in a manner that results
in altered translational decoding by amino acid substitution as “I” base pairs like “G” instead of like “A”. This occurs in RNAs exemplified by those encoding the neurotransmitter receptors GluR-B and 5HT-2cR and in the hepatitis delta virus antigenome RNA (21). RNAs also can undergo non-selective hyperediting as illustrated by MV and polyomavirus RNAs (56). In addition to effects on coding potential, A-to-I editing can destabilize dsRNA structures because product I:U base-pairs generated by editing are less stable than substrate A:U base-pairs (8, 9, 68). The ADAR1 protein is organized in three functional regions. Either one or two Z-DNA binding domains are present at the N-terminal region; three dsRNA binding domains similar to those found in PKR are present in the central region; and, the C-terminal region includes the deaminase catalytic domain (26, 32, 46). ADAR1 is encoded by a single gene, but two size isoforms are produced through the use of alternate promoters and alternative splicing (20, 46). A short form of ADAR1, known as p110, is constitutively expressed and predominantly, if not exclusively, localized in the nucleus; a long, IFN inducible form, known as p150, is found in both the cytoplasm and nucleus (20, 21, 46, 48). The human p150 protein possesses two Z-DNA binding domains, Zα and Zβ (26) and a nuclear export signal (48) within an 295 N-terminal amino acid extension compared to the constitutive p110 protein that initiates at methionine 296 and hence lacks Zα. Both p110 and p150 are active deaminases (9, 20, 37, 46, 56).

PKR and ADAR1 play opposing functional roles in the host innate immune response to MV infection (23, 47, 51, 56). PKR is antiviral and proapoptotic; PKR activation correlates with enhanced IFN-induction (40), increased MAP-kinase pathway activation (40, 61), and reduction in virus growth (64) and increased cytotoxicity and
apoptosis of infected cells (64). ADAR1, conversely, generally acts in a proviral and antiapoptotic manner, at least in cell culture; ADAR1 deficiency leads to enhanced PKR activation (44, 65), increased IFN-induction (25, 33) and enhanced IRF-3 phosphorylation and dimerization (33), whereas ADAR1 sufficiency or ectopic overexpression leads to increased virus growth and suppression of apoptosis (33, 58, 65).

The host responses to virus infection include one that involves translational arrest resulting from phosphorylation and inactivation of eukaryotic initiation factor 2α (eIF2α) by cellular kinases, most commonly PKR (53, 54). Formation of stress granules (SGs) is a mark of the response to infection that results from eIF2α inactivation (27) that can occur during virus-mediated PKR activation (71). SGs, whose formation is triggered when polysomes are disassembled, represent aggregates of translationally silent mRNA, stalled 48S initiation complexes, and multiple RNA binding proteins (RBPs) including the SG markers T-cell restricted intracellular antigen-1 (TIA-1) and Ras GAP SH3-domain binding protein 1 (G3BP1) as well as other RBPs (13, 71). Among the roles played by SGs are the storage of translationally inactive mRNAs under conditions of physiologic stress, RNAs that subsequently are either expressed when the stress is eliminated and translation is restored, or alternatively triaged for degradation by processing bodies if the cells do not survive but undergo apoptosis (13, 29, 71). Several viruses including both positive-stranded and negative-stranded ssRNA viruses and dsDNA viruses are capable of inducing SG formation (35, 36, 42, 50, 59). In some instances infection with wildtype (WT) viruses causes suppression of SG formation, whereas mutants can become efficient inducers of SG formation. For example, influenza A virus A/Puerto Rico/8/34 suppresses SG formation, however when the gene for the
non-structural protein NS1, an inhibitor of PKR, is deleted the mutant virus is capable of
SG induction (31). Similarly, WT vaccinia virus is a poor inducer of formation of SG-like structures termed “anti-viral granules” compared to a mutant deleted for E3L protein (59); E3L, like NS1 is a PKR antagonist (49, 56). Another example is West Nile virus (WNV) where SG formation is lineage dependent; lineage 1 viruses inhibited SGs but lineage 2/1 chimeric viruses induced SGs (15, 18).

Whether or not SGs are induced during MV infection is not known. We therefore wished to test whether MV induced the formation of SG, and whether deletion of either the V or C accessory protein affected the SG response. We furthermore examined whether PKR affected the SG response as anticipated from prior work with other viruses, and more importantly the affect of ADAR1 on the SG response. These studies were possible by combined genetic and biochemical approaches because of the availability of isogenic virus mutants deleted for either V or C protein expression, and because of the availability of human cell clones stably deficient for either PKR or ADAR1 protein expression. Our results revealed that C mutant MV was a robust inducer of SG formation compared to either WT or V mutant virus that were poor inducers, that SG formation was PKR-dependent, and surprisingly that ADAR1, while associated with SG, led to an impairment of SG formation. To gain insight into the functional requirements of ADAR1 for suppression of SG formation, as well as ADAR1 roles IFN-β induction, PKR activation and enhancement of MV growth, a complementation analysis of ADAR1\textsuperscript{kd} cells with WT and mutant forms of ADAR1 was carried out.
MATERIALS AND METHODS

Cells and viruses. Parental HeLa cells and Vero cells were cultured in Dulbecco modified Eagle’s medium (DMEM) supplemented with 5% (vol/vol) fetal bovine serum (HyClone), penicillin (100 μg/ml), and streptomycin (100 units/ml) (GIBCO/Invitrogen) as previously described (75). HeLa cells made stably deficient in either ADAR1 (ADAR1kd) (34, 65) or PKR (PKRkd) (74, 75) by a short hairpin RNA interference silencing strategy and the drug-resistant pSUPER.retro.puro vector control cells (CONkd) were as previously described. Knockdown cells were cultured in DMEM maintenance media with puromycin (1 μg/ml) (Sigma). The recombinant parental Moraten measles virus vaccine strain, herein designated as WT, and isogenic V-deficient (Vko) and C-deficient (Cko) mutants were as described previously (17, 64); they were generously provided by Roberto Cattaneo (Mayo Clinic, Rochester, MN). The recombinant MV includes the gene encoding green fluorescent protein (GFP) inserted downstream of the viral H gene. Infections were carried out at a 50 percent tissue culture dose (TCID₅₀) per cell multiplicity of infection (MOI) of 1 or 3 TCID₅₀/cell as indicated (40, 64). Where indicated, arsenite (Sigma Aldrich) treatment was at 0.5 mM for 1 h.

Western immunoblot analysis. Cells were harvested 24 h after infection, and whole-cell extracts prepared as described (64). The Bradford assay method (Bio-Rad) was used to determine protein concentration. Electrophoretic fractionation of extract protein (20-30 μg) was on either 8% or 10% sodium-dodecyl sulfate polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and the membranes blocked either with 5% milk (wt/vol) in phosphate-buffered saline, or 5% BSA (wt/vol) and 0.1% Tween-20 (vol/vol) in Tris-buffered saline for detection of phosphoproteins. Antibodies for detection of
proteins were: rabbit polyclonal against ADAR1 (46), MV H protein (64), PKR and GFP (Santa Cruz Biotechnology); goat polyclonal against TIA-1 (Santa Cruz Biotechnology); mouse monoclonal against G3BP1 and α-tubulin (Sigma); and rabbit monoclonal against phospho-Thr446 PKR (Epitomics). An Odyssey infrared imaging system (Li-COR) was used for detection of immunoblots (40).

**Immunofluorescence.** Cells were grown on 18 mm glass coverslips in 12-well plates and infected at an MOI of 1 TICD₅₀/cell for 24 h. Fixation was with 10% (v/v) neutral-buffered formalin (Sigma) at room temperature for 15 m. Blocking and permeabilization was with Tris-buffered saline (50 mM Tris pH 7.5, 150 mM NaCl) containing 2% (wt/vol) normal donkey serum (Jackson ImmunoResearch) and 0.2% Triton X-100 (CBP buffer) for 30 m at 37°C. Permeabilized cells then were incubated with primary antibody overnight at 4°C using the following antibody dilutions in CBP buffer: mouse monoclonal anti-G3BP1 1:500 (Sigma); goat polyclonal anti-TIA-1 1:50 (Santa Cruz Biotechnology); rabbit monoclonal anti-phospho-Thr446 PKR 1:100 (Epitomics); rabbit polyclonal anti-ADAR1 1:300 (46), rabbit anti-MV N 1:500 (64) and chicken IgY anti-GFP 1:500 (Molecular Probes). GFP is encoded by the recombinant Moraten vaccine strain of virus used in these experiments. However, because the GFP signal was weakened following preparation of samples for immunofluorescence microscopy, antibody against GFP was used to enhance and stabilize the signal. Incubation of coverslips with donkey secondary antibodies diluted in CBP was at 37°C for 1 h at a 1:200 dilution with the following: Alexa Fluor 350 anti-rabbit or anti-mouse (Molecular Probes); AffiniPure FITC-conjugated anti-chicken (Jackson ImmunoResearch); DyLight
594 anti-rabbit (Jackson ImmunoResearch); and Texas Red-conjugated anti-goat (Jackson ImmunoResearch). Images were obtained using an Olympus IX71 microscope with Q-Capture PRO software (QImaging) or an Olympus IX81 microscope equipped with a Disc Spinning Unit (Olympus) and a 60X oil immersion objective using IPLab software (BD Biosciences). Individual channels were linearly adjusted and overlaid in Adobe Photoshop in RGB for creation of final merged images.

Stress granule quantitation. To determine the number of SG-positive cells, a minimum of four wide-field 40X images were captured per experiment, and a total of ~150-200 cells per experiment were counted. Cells displaying punctate immunofluorescent foci of the G3BP1 SG marker protein were recorded as SG-positive. Percentages were determined as the number of SG-positive cells divided by the total number of cells × 10².

Transient transfections and virus titrations. For complementation of ADAR1kd cells, the indicated ADAR1 expression plasmids were transfected at 2 μg per well of a 6-well plate using Fugene HD reagent (Promega) according to the manufacturer’s instructions and as described previously (61). At 24 h after transfection cells were either infected with MV or left uninfected as indicated. MV yields were determined at 24 h after infection. Virus was titered by a modification of the protocol as described (12). Briefly, cells were subjected to 3 cycles of freeze-thaw; dilutions of virus were then used to infect Vero cells seeded in 96 well plates. Titers were determined by counting GFP fluorescent foci at 48 h post infection. Individually infected cells and syncitia were considered as foci; titers are expressed as focus-forming units per ml.
**Plasmid constructions.** Wildtype (WT) and mutant ADAR1 proteins were expressed using the pcDNA6/V5HisA expression vector (Invitrogen). Constructs were prepared using the cDNA clone previously described (46) and generously provided by A.M. Toth of this laboratory. Briefly, the construction of the plasmids was as follows. The construct expressing the p110 size isoform corresponding to the constitutively expressed form of ADAR1 starting from methionine 296 (M296) was prepared by subcloning the 2,946 bp BamHI-XhoI fragment from pcDNA3.1/HisC-M296-a which was previously described (39). The resulting expression construct, pcDNA6/V5HisA-M296-a (herein referred to as ADAR1 p110 WT), encodes the physiologically relevant “a” splice variant form of the p110 protein (22). The construct for the p150 size isoform of ADAR1 that is IFN inducible and initiates from methionine 1 (M1) was prepared by using the 3,678 bp BamHI-XhoI fragment from the pcDNA3.1/HisC-FL-b (38) plasmid, obtained by digestion with XhoI followed by partial digestion with BamHI. The resulting expression construct, pcDNA6/V5HisA-FL-b (herein referred to as ADAR1 p150 WT), expresses the physiologically relevant “b” splice variant form of the p150 full-length protein (22) and also contains an M296L mutation to prevent expression of the p110 protein by leaky scanning. Catalytic and Z\(\alpha\)-DNA binding mutants of the p150 isoform are referred to herein as p150 cat (H910Q, E912A) and p150 Z\(\alpha\) (Y177A) respectively. For the catalytic mutant, the point mutations H910Q and E912A of the highly conserved C-terminal deaminase catalytic CHAE motif were as described (38). The p150 Z\(\alpha\) (Y177A) mutant was based on the essential tyrosine 177 residue required for Z-DNA binding activity identified by Schade et al. (57). The Y177A mutant was generated using the QuikChange
II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol using the following primer pairs with the mutated bases underlined: (+) 5’- ACTCCGAAGAAAGAAATCAATCGAGTTTTAGCCTCCCTGGCAAAGAAG-3’ and (-) 5’-CTTCTTTGCCAGGGAGGTAAAACTCGATTGATTTCTTTCTCGGAGT-3’. Mutations were confirmed by sequence analysis.

Quantitative real-time PCR. IFN-β transcripts were measured by quantitative real-time PCR (qPCR) as previously described (40). Briefly, 6-well cultures were infected with WT MV or left uninfected. Total RNA was isolated using TRIzol (Invitrogen) at 24 h after infection and from parallel uninfected control cells. Random-primed cDNA was prepared using ~2 µg of RNA and SuperScript II (Invitrogen) according to the manufacturer’s protocol. For the qPCR analyses, the primer pairs and cycle programs for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and IFN-β were as previously described (33). qPCR reactions were performed with reverse transcription templates by using IQ SYBR green Supermix (Bio-Rad) and a Bio-Rad MyIQ real-time qPCR instrument. The IFN-β values were normalized to GAPDH values.
RESULTS

Measles virus deficient in C-protein expression efficiently induces stress granule formation but wildtype virus does not. To test whether measles virus (MV) infection leads to stress granule (SG) formation as a mark of virus infection, we used the Moraten vaccine strain of virus because parental (WT) and isogenic mutants lacking the expression of either the V (V\textsuperscript{ko}) or C (C\textsuperscript{ko}) protein are available and entry of the Moraten strain is via the CD46 receptor present on HeLa cells (43, 64). G3BP1 is a well-established SG associated protein and serves as a marker of SG formation (27, 44, 67).

To determine the expression pattern of G3BP1, HeLa parental cells were left uninfected or were infected with either WT, V\textsuperscript{ko} or C\textsuperscript{ko} virus (Fig. 1). Immunofluorescent microscopy revealed punctate cytoplasmic G3BP1 foci that formed most efficiently in the C\textsuperscript{ko} mutant-infected cells compared to cells infected with either WT or the V\textsuperscript{ko} mutant virus (Fig. 1). In uninfected cells, the G3BP1 signal (white) was homogeneously dispersed throughout the cytoplasm and did not appear punctate. GFP reporter (green), engineered into the MV genome downstream of the H gene, and the MV N protein (red) were used to monitor MV infection (Fig. 1). These results establish that MV induces SG formation and suggest that the MV C protein plays a role during infection that results in down regulation of responses leading to SG formation.

Stress granule formation by measles virus is enhanced by PKR and impaired by ADAR1. The dsRNA-dependent protein kinase PKR and the dsRNA-selective adenosine deaminase ADAR1 are established modulators of the host innate response to infection (23, 25, 33, 56), including during MV infection (33, 33, 40, 64, 65). Clonal HeLa cell lines made stably deficient in either PKR or ADAR1 (PKR\textsuperscript{kd} and ADAR1\textsuperscript{kd}, respectively)
were used in loss of function analyses to assess the roles of PKR and ADAR1 in SG formation. The PKR<sup>kd</sup> cells express less than 5% of PKR as compared to CON<sup>kd</sup> drug control or ADAR1<sup>kd</sup> cells, and the ADAR1<sup>kd</sup> cells express less than 15% of the p110 and p150 forms of ADAR1 as compared to the CON<sup>kd</sup> or PKR<sup>kd</sup> cells (33, 64, 65, 74). SG formation measured by the punctate G3BP1 staining pattern was more efficient in C<sup>ko</sup>-mutant infected than WT virus-infected CON<sup>kd</sup> HeLa cells (Fig. 2A), similar to the results obtained with parental HeLa cells (Fig. 1). Both the parental and the CON<sup>kd</sup> HeLa cells are PKR-sufficient and ADAR1-sufficient. By contrast, SG formation was not detectable in the PKR<sup>kd</sup> cells infected with either C<sup>ko</sup> or WT virus, or in uninfected PKR<sup>kd</sup> or CON<sup>kd</sup> cells, as shown in figure 2A. These results establish a dependency on PKR for formation of SG in MV-infected cells. While SG formation was not readily detectable in CON<sup>kd</sup> cells infected with WT virus, surprisingly WT virus became a potent inducer of SG formation in ADAR1<sup>kd</sup> cells (Fig. 2A). Quantitation revealed less than 3 to 5% of the CON<sup>kd</sup> cells were SG-positive following WT virus infection, but ~50% of the CON<sup>kd</sup> cells were SG-positive following infection with the C<sup>ko</sup> mutant (Fig. 2B). By contrast, a comparably high percentage (~50 to 60%) of the ADAR1-deficient (ADAR1<sup>kd</sup>) cells were SG-positive at 24 h after infection with either WT or C<sup>ko</sup> virus (Fig. 2B). SGs were undetectable in uninfected CON<sup>kd</sup>, PKR<sup>kd</sup> and ADAR1<sup>kd</sup> cells, or in infected PKR<sup>kd</sup> cells (Fig. 2B). Results obtained with G3BP1 were confirmed with TIA-1 as a second marker of the SG. The TIA-1 protein in uninfected cells is largely nuclear in contrast to the cytoplasmic G3BP1 protein, however TIA-1 translocated to the cytoplasm and colocalized with the G3BP1 foci as shown by C<sup>ko</sup> infection of CON<sup>kd</sup> cells (Fig. 2C). These results suggest that
ADAR1 acts to impair SG formation following MV infection; the findings also further suggest that the viral C protein plays a role in modulating the efficiency of SG formation dependent upon the level of ADAR1 protein in PKR-sufficient cells. While the localization of G3BP1 and TIA-1 to foci characteristic of SG was induced by MV infection (Fig. 1, 2), the steady-state expression levels of G3BP1 and TIA-1 were not detectably altered following infection. The three cell types, CON\textsuperscript{kd}, PKR\textsuperscript{kd} and ADAR\textsuperscript{kd} expressed similar levels of both G3BP1 and TIA-1 as assayed by Western immunoblot analysis (Fig. 3A). Infection with MV, either WT, V\textsuperscript{ko} or C\textsuperscript{ko} virus, did not affect the steady-state level of either G3BP1 or TIA-1. By contrast to the enhanced induction of SG in ADAR1-deficient ADAR1\textsuperscript{kd} cells and virtual absence of SGs in PKR-deficient PKR\textsuperscript{kd} cells following MV infection (Fig. 2), arsenite treatment comparably induced the formation of SGs in uninfected CON\textsuperscript{kd}, PKR\textsuperscript{kd} and ADAR1\textsuperscript{kd} cells (Fig. 3B).

**ADAR1 and Phospho-PKR colocalize with MV-induced stress granules.**

Multiple RNA binding proteins (RBPs) colocalize with SG markers (3, 4, 10, 27). Among these, ADAR1, when either ectopically expressed as p150 or endogenously expressed, is described as a component of HeLa cell SG following arsenite treatment or transfection with polyinosinic-polycytidylic acid (pIpC) (69). We wished to test whether a physiologically natural stressor, virus infection, also would cause colocalization of ADAR1 with the induced SG. This possibility was examined with CON\textsuperscript{kd} cells infected with C\textsuperscript{ko} virus, because this cell-virus combination possesses endogenous ADAR1 and SGs are abundantly formed following infection (Fig. 2). At 24 h after infection, cells were analyzed by confocal microscopy using antibodies to G3BP1 (white; blue in merged
images), ADAR1 (red) and GFP (green). Individual channels and merged images are shown in figure 4. The ADAR1 signal detected in uninfected cells not treated with IFN was largely nuclear as anticipated (46) because the ADAR1 p110 isoform is constitutively expressed and is known to display predominantly, if not exclusively, nuclear localization (56). Upon infection with C\(^{ko}\) virus, the ADAR1 signal became punctate in the cytoplasm and colocalized with the G3BP1 marker of SG (Fig. 4A). This colocalization of ADAR1 and G3PB1 was observed in less than 10% of cells (data not shown). Our finding that ADAR1 localizes to the MV-induced SG (Fig. 4A) is in agreement with the recent reports using either the non-physiologic pIpC inducer of stress (69) or hepatitis C virus infection (50). However, the presence of ADAR1 protein with SG does not appear to be an obligatory requirement for maintenance of SGs, but rather unexpectedly the converse; the presence of SG structures was substantially increased in ADAR1-deficient cells following MV infection compared to ADAR1-sufficient cells (Fig. 2).

We also examined whether the dsRNA-dependent kinase PKR, following activation by phosphorylation, colocalized with MV-induced SG. At 24 h after C\(^{ko}\) infection of CON\(^{kd}\) cells, confocal microscopy was carried out using antibodies for G3BP1 (white; blue in merged image), phospho-Thr446-PKR (red) and GFP (green). The phospho-PKR signal was weak in uninfected cells, but following MV infection the signal was greatly increased within the cytoplasm, characteristic of activation of PKR (Fig. 4B). Furthermore, the staining pattern of phospho-PKR appeared punctate in part and the punctate signal colocalized with the G3BP1 marker of SG.
Deaminase catalytic activity of the p150 ADAR1 protein is required for suppression of MV-induced host responses including stress granule formation. We earlier found that ADAR1 acts as a suppressor of PKR activation during MV infection (33, 65). In ADAR1-deficient cells, PKR activation is enhanced compared to ADAR1-sufficient cells. As was shown in figure 2, ADAR1-deficient cells displayed enhanced SG formation after infection with WT MV. To probe the mechanistic basis of the suppression by ADAR1 of SG formation (Fig. 2) and also PKR activation and IFN-β induction (33, 65), we used a complementation strategy. We tested the functional effects of ectopic expression of different forms of ADAR1 that included the WT p110 and WT p150 ADAR1 proteins, and mutant p150 proteins defective in either the deaminase catalytic or Z-DNA binding activities. SG formation and PKR activation as well as IFN-β transcript production were examined in complemented ADAR1kd cells, both uninfected and MV-infected (Fig. 5).

Uninfected (UI) ADAR1kd cells, either complemented or not by ectopic expression, did not show detectable SGs measured by G3BP1 staining (Fig. 5A, left). However, MV-infected ADAR1kd cells (Fig. 5A, right) showed robust SG formation when they either had not been transfected (ADAR1kd) or when they had been complemented by expression of the p110 (WT) ADAR1 protein or transfected with the empty vector as a control. However, cells complemented with either the p150 (WT) protein or the p150 Z-DNA binding defective mutant Y177A showed low levels of SG formation following infection (Fig. 5 A,B). By contrast, the p150 catalytic mutant H910Q, E912A showed high levels of SG formation (Fig. 5 A,B). These results suggest that the p150 size isoform of ADAR1, and furthermore catalytic activity of the p150 protein, are required to suppress SG formation following infection with WT MV.
Because ADAR1 is known to function as a suppressor of PKR activation (23, 56, 65), PKR phosphorylation at Thr446 as a measure of PKR activation was determined in ADAR1-complemented ADAR1kd cells (Fig. 5C). Only the p150 (WT) and p150 Zα mutant constructs were able to significantly suppress PKR activation (Fig. 5C,D).

Because ADAR1 also is implicated as a suppressor of IFN induction (25, 33), induction levels of IFN-β transcripts were determined. As shown by the representative data set in figure 5E, ADAR1kd cells expressing either p150 (WT) or the p150 Zα mutant suppressed IFN-β RNA expression to levels approaching that seen with CONkd cells (Fig. 5E). By contrast, complementation of ADAR1kd cells with either p110 (WT) or the p150 catalytic mutant did not decrease the level of either phospho-PKR (Fig. 5D) or IFN-β RNA (Fig. 5E) from that seen in ADAR1kd cells either not complemented or transfected with empty vector. These results suggest that deaminase catalytic activity of the ADAR1 p150 protein is required for suppression of both PKR activation and IFN-β induction.

Because PKR is the eIF2α kinase activated following MV infection, ADAR1 suppression of PKR activation emerges as a likely mechanism by which ADAR1 deficiency contributes to enhanced SG formation, in part because of enhanced PKR activation, just as we recently established for induction of IFN-β (33).

**Wildtype p150 ADAR1 but not the catalytic mutant enhances MV growth.** ADAR1 displays a proviral effect with a range of RNA viruses (23, 56). The mechanism remains unclear. To gain insight into the functional domains of ADAR1 required, and whether the enhancement of MV growth by ADAR1 correlates with the suppression of SG formation, we again employed the complementation strategy. The results are shown in figure 6.

Enhancement of WT MV growth in ADAR1kd cells was only observed in the cells
complemented to express either the p150 (WT) or the p150 Zα mutant (Fig. 6). Virus yields in the ADAR1kd cells expressing either the p110 (WT) or p150 catalytic mutant were low and comparable to ADAR1kd cells that either had not been complemented or had been transfected with empty vector (Fig. 6).
Our studies were undertaken with the aim of testing the roles of PKR and ADAR1, two dsRNA binding proteins, in virus-induced stress granule formation following MV infection, and whether the viral C and V accessory proteins affect the SG response. By using cells stably deficient either in PKR (PKR<sup>kd</sup>) or ADAR1 (ADAR1<sup>kd</sup>), we established that SG formation was induced by MV infection in a PKR-dependent manner. But conversely, virus-induced SG formation was impaired by ADAR1. Furthermore, MV deficient in C protein expression was a more efficient inducer of SG formation than either WT or the isogenic V-deficient mutant. Complementation analyses provided mechanistic insight. Ectopic expression of ADAR1 in cells deficient of both p110 and p150 ADAR1 proteins established that the deaminase catalytic activity of the ADAR1 p150 isoform was necessary for suppression of SG formation. p150 ADAR1 catalytic activity was also necessary for suppression of PKR activation and IFN-β induction. Several important points emerge from these findings that extend prior observations. First, we found that following infection with either WT or V<sup>ko</sup> virus SGs were not efficiently induced in PKR-sufficient, ADAR1-sufficient HeLa cells, either parental cells or CON<sup>kd</sup> control cells (<5% SG-positive). However, induction of SG in these cells after infection with C<sup>ko</sup> virus was striking: ~50 to 60% of the control cells became SG-positive following infection with the mutant virus. The finding that SG-positive PKR<sup>kd</sup> cells were virtually undetectable following infection with measles virus is consistent with recent observations for West Nile virus, hepatitis C virus, influenza virus and respiratory syncytial virus (15, 31, 36, 50), where SG formation induced by these viruses also was PKR dependent. Because SG formation is dependent on eIF2α phosphorylation (4, 27),
these findings further suggest that PKR is the predominant eIF2α kinase responsible for SG formation by these viruses rather than PERK activated by endoplasmic reticulum stress, HRI activated by oxidative stress and heavy metals or GCN2 activated by nutritional stress, all of which also are eIF2α kinases (30, 54).

By contrast to the PKR dependency for SG formation with MV, we observed in cells deficient for ADAR1 that SG formation was far more efficient than in ADAR1-sufficient cells infected with WT virus. Furthermore, the SG formation seen with WT and C<sup>ko</sup> virus in the ADAR1<sup>kd</sup> cells was comparable to the same high level seen in CON<sup>kd</sup> cells infected with C<sup>ko</sup> virus. The impairment of SG formation by ADAR1 in WT virus-infected CON<sup>kd</sup> cells was striking. These results suggest that ADAR1 was capable of suppression of SG formation induced by WT virus infection but not with C<sup>ko</sup> infection.

What then is the mechanistic basis of these novel effects? Does ADAR1 act as an enzyme, or as an RNA-binding protein similar to G3BP1, TIA-1 or Staufen (60, 72), or possibly as both to mediate the suppression of SG formation?

To begin to address the question of the functional activity and size isoform of ADAR1 required to mediate the suppressing effects, we employed a complementation strategy by expression WT and mutant forms of ADAR1 in the ADAR1<sup>kd</sup> cells. The results established that deaminase catalytic activity of the p150 size isoform was required; the p110 size isoform of ADAR1 was unable to suppress either SG formation or activation of PKR following MV infection. The p150 size isoform is IFN inducible and is the only known ADAR protein found in the cytoplasm, whereas the p110 size isoform is constitutively expressed and predominantly if not exclusively nuclear (46, 55). Both p150 and p110 are efficient dsRNA binding proteins that possess three copies of the
prototypical dsRNA-binding domain first identified in PKR (38, 46, 56). Because p110 is unable to rescue the ADAR1kd phenotype, RNA binding activity of ADAR1 alone appears insufficient for rescue because the three RNA-binding domain copies are identical between p110 and p150. In addition to the p150 WT protein, the p150 Zα mutant also rescued the SG phenotype. The Zα domain of ADAR1 p150 was mutated for two reasons: firstly, Zα is unique to p150 and is not present in the p110 protein (6, 7); and secondly, another Z-DNA binding protein, ZBP1, that has two Zα domains is reported to localize to SGs upon heat shock and arsenite treatment (16). Our results indicate that ADAR1 suppression of SG formation requires A-to-I editing activity but not Z-DNA binding activity of Zα.

While MV infection induced SG formation as measured by the appearance of punctate cytosolic staining patterns of two beacons of SG, the G3BP1 and TIA-1 RNA-binding proteins, virus infection did not alter the steady-state level of either G3BP1 or TIA-1, only the aggregation of them in the cytoplasm. The levels of TIA-1 and G3BP1 were comparable in the three engineered cell lines, PKRkd, ADAR1kd and CONkd cells, both uninfected and infected for 24 h with WT or Vko or Cko mutant virus. MV-induced SG characterized by the TIA-1 and G3BP1 markers also contained ADAR1 and phospho-PKR. However, the SGs did not contain MV proteins (N or P) to a detectable level (Fig. 1 and data not shown). Our finding that ADAR1 was associated with SG following MV infection is in agreement with recent reports that likewise showed endogenous ADAR1 associated with SG, either following infection with HCV as the stress inducer (50) or ectopically expressed ADAR1 p150 following treatment with arsenite or transfection with synthetic pIpC dsRNA as the stress inducer (69). Curiously the cellular protein
Staufen displays some characteristics similar to ADAR1. Staufen is a dsRNA binding protein (60) and is SG-associated (1, 62, 63). Overexpression of Staufen leads to decreased SG formation, while transient knock-down of Staufen leads to enhanced SG formation (63). Likewise, overexpression of p150 (WT) ADAR1 led to decreased SG formation, whereas stable knock-down of ADAR1 enhanced SG formation following infection. These results suggest that both ADAR1 and Staufen, while not essential components of induced SGs, yet they modulate the efficiency of SG formation.

Enhanced PKR activation and induction of IFN-β also are observed in ADAR1<sup>kd</sup> cells infected with MV (33, 65). Enhanced levels of PKR activation likewise are observed in vesicular stomatitis virus-infected ADAR1-deficient cells compared to ADAR1-sufficient cells in culture (34, 44), and genetic disruption of ADAR1 in mice results in up regulation of IFN expression in hematopoietic stem cells (25). The robustness of SG formation that we observed herein correlated with the extent of PKR activation and also IFN-β induction. While it is tempting to speculate that formation of SG may play a role in antiviral innate signaling triggered by MV including IFN-β induction, further analyses are necessary to establish more definitively or eliminate this possibility. ADAR1 suppressed virus-induced host responses seen with WT but not C<sup>ko</sup> mutant virus include, in addition to suppression of PKR activation (33, 65) and IFN-β induction (33, 65), also the suppression of SG formation as shown herein. ADAR is known to destabilize dsRNA structures (9) and dsRNA is a potent activator of PKR and inducer of SG formation (4, 5, 52, 71). The requirement for ADAR1 catalytic activity for rescue of the ADAR1<sup>kd</sup> phenotype is consistent with a role of ADAR1 as a dsRNA destabilizer, as I:U mismatch base-pairs are less stable than A:U pairs (9).
The difference in capability of WT compared to the C<sup>ko</sup> mutant to induce SG formation was striking. Among the possible explanations, perhaps the C protein of WT MV modulates viral RNA synthesis in a quality control manner to minimize aberrant RNA formation such as that seen with human parainfluenza virus type 1 (11). In the absence of C protein, aberrant viral RNAs with sufficient structure to activate PKR and trigger SG formation might accumulate to such an elevated concentration that the endogenous ADAR1 is unable to destabilize them. Thus, ability of C protein expressed by WT to effectively down regulate innate host responses may be an indirect consequence during virus replication. With viruses other than WT measles virus, SG formation is sometimes impaired and multiple mechanisms may be operative (71). These range from direct antagonism of PKR and eIF2α phosphorylation by viral gene products exemplified by the NS1 protein of influenza virus (31) and E3L protein of vaccinia virus (59) to the cleavage or sequestration of RNA-binding proteins that drive SG formation exemplified by cleavage of G3BP1 by the 3C protease of poliovirus (70) or depletion of Staufen by the HIV gag protein through direct interaction (1).

Taken together, our results establish that SG formation induced by MV infection is modulated in an opposing manner by ADAR1 and PKR. SG formation is impaired by ADAR1, just as the activation of PKR and induction of IFN-β likewise are impaired by ADAR1 (33, 65). But MV-induced SG formation is dependent upon PKR. The opposing roles of ADAR1 and PKR suggest that these proteins may likely contribute to the dynamic oscillation of SG formation characteristic of the cellular response to virus infection (2, 28, 50). It is now of utmost importance to establish the qualitative and quantitative nature of the RNA produced in MV-infected cells that is responsible for
PKR-dependent SG formation, and whether SG formation *per se* is necessary for triggering an antiviral innate immune response characterized by IFN induction and action. The data presented herein further illustrate the delicate balance between PKR and ADAR1, and add to a growing body of evidence that despite being an IFN-stimulated gene, ADAR1 in some instances both suppresses host responses characteristic of virus infection and enhances virus growth (23, 56).
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Measles virus deficient for C-protein expression is an efficient inducer of stress granule formation but wildtype virus is not. Cells were either left uninfected (UI) or infected with WT, \(V^{ko}\) or \(C^{ko}\) MV at a multiplicity of infection of 3 TICD\(_{50}\)/cell. At 24 h after infection cells were analyzed by immunofluorescence microscopy as described under Materials and Methods using antibodies to G3BP1 (white) as a marker for SG formation, and N (red) and GFP (green) for detection of MV infection. Individual channels are shown.

Figure 2. Stress granule formation is impaired by PKR-deficiency and amplified by ADAR1-deficiency following measles virus infection. (A) PKR\(^{kd}\), ADAR1\(^{kd}\) and \(CON^{kd}\) cells were either left uninfected (UI) or infected with WT or \(C^{ko}\) mutant MV as indicated. At 24 h after infection cells were analyzed by immunofluorescence microscopy as described under Materials and Methods using antibodies to G3BP1 (red) as a marker for detection of SG formation and GFP (green) for detection of MV infection. Images shown are merged. (B) Quantitation of SG-positive cells. Wide-field 40X images were obtained and a minimum of 150 cells were counted per experiment. Results shown are means and standard errors of independent experiments. **, \(P < 0.0005\) by Student’s \(t\)-test comparing WT infected cells to \(C^{ko}\) infected cells to infected cells. (C) \(CON^{kd}\) cells were infected with \(C^{ko}\) MV or left uninfected (UI). At 24 h after infection cells were analyzed by confocal microscopy using antibodies against G3BP1 (white), TIA-1 (red) and GFP (green). Individual channels and merged images are shown; G3BP1 appears blue in merged images.
Figure 3. Stress granule marker proteins G3BP1 and TIA-1 are comparably expressed in uninfected and measles virus-infected CONkd, PKRkd and ADAR1kd cells. (A). Cells were either left uninfected (UI) or infected with WT, Vko or Cko MV at a multiplicity of infection of 3 TICD₅₀/cell. At 24 h after infection whole-cell extracts were prepared and analyzed by Western immunoblot assay with antibodies against ADAR1, PKR, G3BP1, TIA-1, MV H, GFP and α-tubulin as described under Materials and Methods. (B) Uninfected cells treated with arsenite as described under Materials and Methods were analyzed by immunofluorescence microscopy using antibody against G3BP1.

Figure 4. ADAR1 and PKR colocalize with stress granules induced by infection with measles virus. CONkd cells were infected with Cko mutant MV and analyzed at 24 h post infection by confocal microscopy using antibodies against G3BP1 (white), GFP (green), and either (A) ADAR1 (red) or (B) phospho-Thr446-PKR (red). Individual channels and merged images are shown; G3BP1 appears blue in merged images.

Figure 5. ADAR1 deaminase activity of the p150 protein isoform is required for suppression of stress granule formation as well as suppression of PKR activation and IFN-β induction following measles virus infection. ADAR1kd cells deficient in p150 and p110 were complemented by transfection with ADAR1 constructs or the vector plasmid as indicated, or ADAR1kd and CONkd cells were also left untransfected as controls. At 24 h after transfection, cells were either infected with WT measles virus.
(MV) or left uninfected (UI). Infection was with a multiplicity of infection of 1 TICD₅₀/cell for microscopy experiments or 3 TICD₅₀/cell for Western immunoblot assays, IFN-β mRNA quantitation and yield determinations. ADAR1⁴ cells complemented with ADAR1 proteins are designated p110 (WT) and p150 (WT) for wildtype protein isoforms, and p150 Zα (Y177A) and p150 catalytic (H910Q, E912A) for p150 proteins with mutated domains. Vector, empty vector. (A) At 24 h after infection cells were analyzed by immunofluorescence microscopy using G3BP1 antibody as a marker for SG formation; (B) Quantitation of SG-positive cells. Wide-field 40X images were obtained and a minimum of 150 cells were analyzed per experiment for the presence of stress granules as shown in (A). *, P < 0.001 **, P ≤ 3x10⁻⁵ by Student’s t-test comparing uninfected ADAR1⁴ cells to infected cells. The results shown are means and standard errors of three independent experiments. (C) Western immunoblot analysis of ectopic ADAR1 expression. At 24 h post infection, whole-cell extracts were prepared and analyzed using antibodies against ADAR1, PKR and phospho-Thr446-PKR and α-tubulin as indicated. (D) Quantitation of PKR activation (n-fold) as measured by the level of phospho-Thr446-PKR to total PKR protein determined by Western immunoblot analysis as shown in panel C. *, P < 0.05 by Student’s t-test to compare the level of P-PKR in infected ADAR1⁴ cells to infected cells complemented as indicated, or to CON⁴ cells. The results shown are means and standard errors of three independent experiments. (E) IFN-β mRNA levels. At 24 h after infection total RNA was isolated and IFN-β transcript levels were determined by qPCR and normalized to GAPDH. Results shown are representative of 4 independent experiments.
Figure 6. ADAR1 p150 wildtype but not catalytic mutant protein enhances measles virus growth. ADAR1<sup>kd</sup> cells were complemented as described under the legend for figure 5. The yield of WT virus was measured at 24 h post infection as determined under Materials and Methods; titers are expressed as fluorescent focus units per ml on Vero cells. *, P < 0.02 by Student’s t-test. Results shown are means and standard errors of four independent experiments.


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Figure 1
Figure 2A
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Figure 4
Figure 5A

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Figure 5B

% SG positive cells

- CON

- ADAR1

- p110 (WT)

- p150 (WT)

- p150 (Zα) (Y177A)

- p150 (cat) (H910Q,E912A)

- Vector
Figure 5D

PKR activation (fold)

- CON
- ADAR
- P10 (WT)
- P150 (WT)
- P150 (Zδ)
- P150 (Gal)
- Vector

* Significant difference.
Figure 5E

IFN-β induction (fold)

0  500  1000  1500  2000  2500  3000  3500  4000

CON

ADAR1

p110 (WT)

p150 (WT)

p150 (Zc)

(p177)

p150 (cat)

(H2100.E312A)

vector