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RNA adenosine deaminase ADAR1 deficiency leads to increased activation of protein kinase PKR and reduced vesicular stomatitis virus growth following interferon treatment

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ABSTRACT

Two size forms of ADAR1 adenosine deaminase are known, one constitutively expressed (p110) and the other interferon (IFN)-induced (p150). To test the role of ADAR1 in viral infection, HeLa cells with ADAR1 stably knocked down and 293 cells overexpressing ADAR1 were utilized. Overexpression of p150 ADAR1 had no significant effect on the yield of vesicular stomatitis virus. Likewise, reduction of p110 and p150 ADAR1 proteins to less than ~10 to 15% of parental levels (ADAR1-deficient) had no significant effect on VSV growth in the absence of IFN treatment. However, inhibition of virus growth following IFN treatment was ~1 $\log_{10}$ further reduced compared to ADAR1-sufficient cells. The level of phosphorylated protein kinase PKR was increased in ADAR1-deficient cells compared to ADAR1-sufficient cells following IFN treatment, regardless of viral infection. These results suggest that ADAR1 suppresses activation of PKR and inhibition of VSV growth in response to IFN treatment.

Introduction

Adenosine deaminase acting on RNA (ADAR1) is an RNA editing enzyme that catalyzes the C-6 deamination of adenosine (A) to generate inosine (I) in RNA substrates that possess double-stranded RNA character (Samuel, 2001; Bass, 2002; Valente and Nishikura, 2005; Toth et al., 2006). A-to-I editing is of broad physiologic significance, because I is recognized as G instead of A by ribosomes during translational decoding of mRNA and also by polymerases during RNA-dependent transcription (Bass, 2002; Toth et al., 2006). Such substitution A-to-I editing is seen in both cellular and viral RNAs. The deamination editing can be site-selective, occurring at one or a few A's, thereby generating protein products with altered function because of selective amino acid substitutions arising from the substitution of A with I (G). Among the best characterized examples of selective editing specified by imperfect duplex RNA structures are the hepatitis delta virus antigenome RNA (Luo et al., 1990; Jayan and Casey, 2002) and the cellular pre-mRNAs for the L-glutamate (GluR) and serotonin-2c (5-HT_{2c}) receptors in the nervous system (Higuchi et al., 1993; Liu and Samuel, 1999; Liu et al., 1999; Maas et al., 2001; Seeburg and Hartner, 2003).

DsRNA-specific deamination by ADAR1 also can occur at multiple sites within perfect duplex RNA substrates (Liu and Samuel, 1996; Kumar and Carmichael, 1997; Liu et al., 2000). Indeed, dsRNA-specific adenosine deaminase enzymatic activity was first described as a developmentally regulated dsRNA duplex-unwinding activity in *Xenopus* oocytes (Rebagliati and Melton, 1987; Bass and Weintraub, 1988). But now it is recognized that, rather than unwinding duplex dsRNA to separate strands, the RNA becomes more single-stranded in character because stable A:U base pairs are changed to less stable I:U base pairs (Bass and Weintraub, 1988; Wagner et al., 1989).

The *ADAR1* gene is single copy, ~40-kbp with 17 exons, and maps to human chromosome 1q21 (Weier et al., 1995; Liu et al., 1997). ADAR1 is interferon-inducible (Patterson et al., 1995; George and Samuel, 1999; George et al., 2008). Two size forms of the ADAR1 protein are known, an IFN-inducible protein of ~150-kDa designated p150 that is found in both the cytoplasm and nucleus, and a constitutively expressed protein of ~110-kDa designated p110 that is predominantly if not exclusively a nuclear protein (Patterson and Samuel, 1995; Toth et al., 2006). At least three alternative promoters, one of which possesses an ISRE element and is IFN-inducible, together with alternative splicing, drive the expression of *ADAR1* transcripts with alternative exon 1 structures (George and Samuel, 1999; Kawakubo and Samuel, 2000; George et al., 2005, 2008). Translation initiation of the IFN-inducible 1200 amino acid protein (p150) begins at AUG1 present in the alternative exon 1A at the 5′-termini of IFN-inducible transcripts, whereas the alternative exon 1B and 1C structures at the 5′-termini of constitutively expressed ADAR1 transcripts

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both lack AUGs; translation initiation of the constitutively expressed 931 amino acid protein (p110) begins at the in-frame AUG296 present in exon 2 (George and Samuel, 1999; Valente and Nishikura, 2005; Toth et al., 2006). A second ADAR gene, *ADAR2*, maps to human chromosome 21q22 and encodes an ~80-kDa RNA adenosine deaminase that is a nuclear protein. ADAR2 is not regulated by IFN (Melcher et al., 1996; Villard et al., 1997; Toth et al., 2006).

Both p150 and p110 are active ADAR1 deaminases (Toth et al., 2006). The p150 and p110 proteins possess, in addition to the deaminase catalytic domain present in their C-terminal region, three copies of a dsRNA-binding motif in the central region that is homologous to the dsRNA-binding motif first discovered in the RNA-dependent protein kinase PKR (Kim et al., 1994; O'Connell et al., 1995; Patterson and Samuel, 1995; Liu and Samuel, 1996). The IFN-inducible p150 protein is N-terminally extended compared to p110, and possesses two copies of a Z-DNA binding motif (Patterson and Samuel, 1995; Athanasiadis et al., 2005). Two viral interferon resistance gene products that antagonize PKR kinase activity, the vaccinia virus E3L protein (Chang and Jacobs, 1993; Zhang et al., 2008) and the adenovirus VAI RNA (Kitajewski et al., 1986), also antagonize ADAR1 deaminase activity (Lei et al., 1998; Liu et al., 2001).

PKR is well established as an IFN-inducible antiviral protein and an important component of the IFN innate antiviral defense system (Haller et al., 2006; Toth et al., 2006; Sadler and Williams, 2008). PKR is a cytoplasmic RNA sensor; dsRNA and structured single-stranded RNAs are bound by PKR and mediate autophosphorylation and activation of PKR (Samuel, 1993). The best characterized substrate of PKR is the α subunit of translation initiation factor eIF-2, which when phosphorylated on serine 51, leads to an inhibition of protein synthesis in IFN-treated infected cells (Samuel, 1979; García et al., 2006; Sadler and Williams, 2008). Among IFN sensitive viruses is vesicular stomatitis virus (VSV), a negative-strand RNA virus widely used in IFN antiviral studies (Samuel, 2001; Haller et al., 2006; Randall and Goodbourn, 2008). Type I IFNs induce an antiviral state against VSV characterized by an inhibition of VSV protein production by a mechanism in which the PKR kinase plays an important role (Masters and Samuel, 1983; Balachandran et al., 2000; García et al., 2006).

Because PKR is activated by dsRNA, and because ADAR1 acts on dsRNA, to test whether ADAR1 affects PKR activation and VSV growth, cells in which >85% of both basal and IFN-inducible ADAR1 protein expression was stably silenced using a short hairpin RNA interference approach were examined. By comparing wild-type parental HeLa cells and control knockdown HeLa cells that are ADAR1-sufficient with the ADAR1-deficient knockdown cells, we found that ADAR1 loss did not affect the growth of VSV in the absence of IFN treatment. Likewise, overexpression of either ADAR1 or ADAR2 did not affect VSV single-cycle growth in 293 cells. However, in IFN-treated cells, the loss of ADAR1 led to enhanced activation of PKR and reduced yields of VSV.

Results

Overexpression of ADAR does not affect vesicular stomatitis virus growth

The single-cycle growth of VSV was examined in HEK 293 cell lines engineered to overexpress either ADAR1 p150 or ADAR2 protein to levels ~7 to 8 fold higher than that of parental 293 cells (Maas et al., 2001). As shown in Table 1, neither ADAR1 nor ADAR2 overexpression significantly affected VSV multiplication compared to parental 293 cells. The single-cycle yields at 24 h after infection of ADAR1 or ADAR2 overexpressing cells (2800 and 3200 PFU/cell, respectively) differed by only ~two-fold from that of the control parental 293 cells (6500 PFU/cell). Because it was possible that multiple rounds of replication might be necessary in order to accumulate sufficient mutations resulting from A-to-I editing to cause a measurable effect on the yield of infectious progeny, we then passed the virus for ten rounds of growth in 293 cells that stably overexpressed ADAR1 or ADAR2 as well as in control 293 cells. In all three 293 cell lines, the yield of infectious progeny remained relatively unchanged for three passages, decreased during passages 4 and 5, and then increased during passage 6 and began cycling (data not shown), a phenomenon described earlier for VSV (Huang and Baltimore, 1970; Palma and Huang, 1974).

Table 1
Stable overexpression of ADAR1 p150 or ADAR2 does not affect the single-cycle yield of vesicular stomatitis virus[a].

Cell	Time after infection (hours)		
	4	6	24
	(PFU/cell)	(PFU/cell)	(PFU/cell)
HEK293	220	2200	5600
293-ADAR1wt	420	1000	2800
293-ADAR2wt	520	1600	3200

[a] Virus yields were measured with the Indiana serotype of VSV in parental HEK 293 cells (HEK 293), and HEK 293 cell clones stably overexpressing either recombinant ADAR1 p150 (293-ADAR1wt) or ADAR2 (293-ADAR2wt) wild-type protein. Cells were harvested at the indicated times after infection, and virus yields determined by plaque titration on mouse fibroblast L cells.

Stable knockdown of cytoplasmic and nuclear ADAR1 proteins

Because overexpression of ADAR1 did not significantly affect VSV multiplication, we next wished to test the effect of ADAR1 p110 and p150 deficiency on virus growth. The HeLa cell clonal line 132 was examined in which a short hairpin-based RNA interference strategy was utilized to silence ADAR1 gene expression (Toth et al., 2009). As measured by Western analysis of whole-cell extracts (Fig. 1A), the

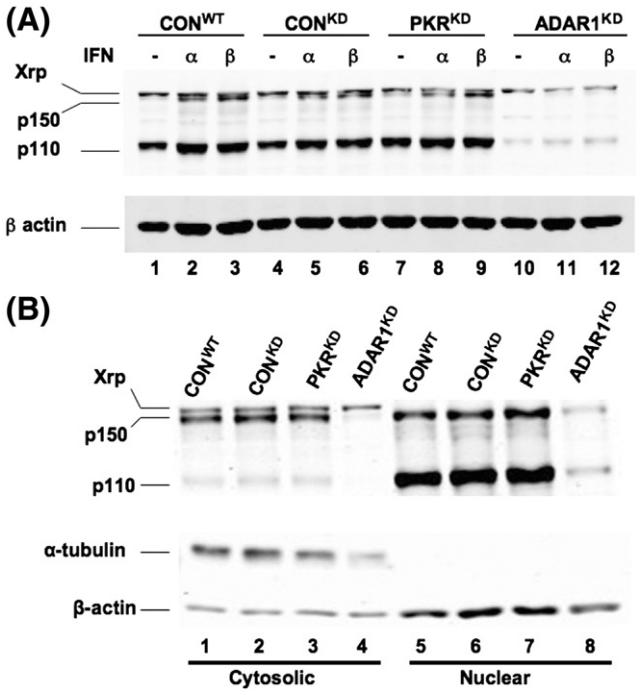

Fig. 1. Stable knockdown of ADAR1 proteins. Western immunoblot analyses comparing ADAR1 expression in wild-type parental (CON^{WT}), ADAR1 stable knockdown ($ADAR1^{KD}$), puromycin-resistant control (CON^{KD}) and PKR stable knockdown (PKR^{KD}) HeLa cell clones. The large (150 kDa) and the small (110 kDa) size forms of ADAR1 are designated p150 and p110, respectively. Xrp, a non-specific cross-reacting protein migrating just above p150. (A) Whole-cell extract analysis: cells were mock treated or treated with either 1000 units/ml of IFN-αA/D or IFN-β for 24 h. Whole-cell extract protein (10 μg) was analyzed in each lane on SDS–10% PAGE. (B) Analysis of fractionated cell lysates. Cytosolic and nuclear extract fractions were prepared from cells that had been treated with IFN-β 24 h and analyzed on SDS–7% PAGE. Membranes were probed using a polyclonal antibody against human ADAR1 and monoclonal antibodies against β-actin and α-tubulin as loading and fractionation controls (Ahn et al., 2004).