FRET Analysis of In Vivo Dimerization by RNA Editing Enzymes*
Kaari A. Chilibeck, Tao Wu, Chao Liang, Matthew J. Schellenberg, Emily M. Gesner, Jeffrey M. Lynch, and Andrew M. MacMillan§
From the Department of Biochemistry, University of Alberta, Edmonton, Alberta Canada, T6G 2H7
Running Title: FRET Analysis of ADAR Dimerization
§Address correspondence to: Andrew M. MacMillan, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7, Tel. 780-492-3805; Fax. 780-492-0886; Email: andrew.macmillan@ualberta.ca

Members of the ADAR (adenosine deaminase that acts on RNA) enzyme family catalyze the hydrolytic deamination of adenosine to inosine within double-stranded RNAs—a poorly understood process which is critical to mammalian development. We have performed fluorescence resonance energy transfer (FRET) experiments in mammalian cells transfected with fluorophore-bearing ADAR1 and ADAR2 fusion proteins to investigate the relationship between these proteins. These studies conclusively demonstrate the homodimerization of ADAR1 and ADAR2 and also show that ADAR1 and ADAR2 form heterodimers in human cells. RNase treatment of cells expressing these fusion proteins changes their localization but does not affect dimerization. Taken together these results suggest that homo- and heterodimerization are important for the activity of ADAR family members in vivo and that these associations are RNA independent.

Double-stranded RNAs (dsRNAs) in eukaryotes are subject to a variety of processing reactions including cleavage by the RNase III family members Drosha and Dicer in the miRNA and siRNA gene silencing pathways and editing by members of the ADAR (adenosine deaminase that acts on RNA) enzyme family (1, 2). This latter reaction involves the hydrolytic deamination of adenosine (A) to inosine (I) within the context of dsRNA. Editing events of this type have been demonstrated in both cellular and viral transcripts and have been shown to function in altering the coding properties of the edited RNAs. For example, the life-cycle of the Hepatitis δ virus is regulated by an editing event in the anti-genome in which a UAG stop codon is converted to a UIG tryptophan codon (3). An A to I modification is involved in the functional regulation of a growing number of cellular factors. These include the tissue-specific editing of the serotonin 5-HT2C receptor which results in a reduction in response to serotonin agonists (4). Transcripts for sub-units of the neural-specific AMPA class of glutamate-gated (GluR) ion channels undergo A to I modification at two positions, the Q/R and R/G editing sites, which affect the properties of the resulting channels (5, 6). In addition to the editing of these and other neuronal transcripts to effect codon changes, one deaminase family member, ADAR2, has been shown to auto-regulate its own expression by the creation of a 3′ splice site (CAΔ to CAI) (7). Despite the identification of these editing substrates, the global role of A to I modification in higher eukaryotes remains unclear. Measurement of inosine levels in RNA isolated from rat tissue suggests a greater level of editing than indicated by known RNA substrates (8). A cloning protocol which depended upon an inosine-specific cleavage of RNA detected a large number of editing sites in non-coding regions of RNAs from C. elegans and humans which included sites in 5′ and 3′ UTRs and introns (9). Recent bioinformatic studies have suggested the presence of more than 12,000 editing sites corresponding to non-coding regions of the human genome (10-13). The importance of editing in development has been demonstrated by studies showing that deletion of ADAR1 is an embryonic lethal event in mice; fibroblasts derived from ADAR1-/- embryos are subject to stress induced apoptosis (14). Finally, it has been suggested that A to I modification antagonizes RNA interference: editing could regulate endogenous miRNA mediated gene-silencing or limit RNAi resulting from non-specific anti-sense transcription (15-17).

One difficulty in identifying the biological targets and hence roles of A to I editing is that little is understood about the sequence determinants for editing of known substrates beyond the fact that editing sites are found within dsRNAs. The first
RNA deaminase to be cloned, ADAR1 (18-21), was originally identified as an activity responsible for unwinding double-stranded RNA (22-24); subsequently, this unwinding activity was correlated with deamination of A to I within these RNAs (20). Constitutively expressed ADAR1 is a 110 kDa nuclear protein that contains three N-terminal double-stranded RNA binding domains (dsRBDs), a C-terminal deaminase domain, and one N-terminal Z-DNA binding domain. ADAR1 exhibited low deaminase activity with a number of specific substrates, including the Q/R site of the GluR-B pre-mRNA, but has been shown to efficiently edit the R/G site of GluR-B as well as the anti-genome of Hepatitis D virus (3, 26-28).

Screening of a rat hippocampal cDNA library with probes complementary to the deaminase domain of ADAR1 resulted in the cloning of ADAR2 deaminase (29). The shorter of two isoforms, ADAR2a, is an ~80 kDa protein containing two N-terminal dsRBDs as well as a C-terminal deaminase domain (30). ADAR2a (henceforth ADAR2) has been shown to efficiently edit both the Q/R and R/G sites of the Glur-B transcript and in contrast to ADAR1 does not exhibit activity at a position within intron 11 of GluR-B (29). ADAR1 and ADAR2 are both expressed ubiquitously although ADAR2 is enriched in the brain.

During a study of the in vitro editing of the Glur-B R/G site by ADAR2, we observed that efficient editing required dimerization of the enzyme on the RNA substrate (31). This conclusion was based on a combination of kinetic and gel mobility shift analyses as well as RNA-dependent ADAR2•ADAR2 crosslinking. Subsequently, it was reported that recombinant, tagged human ADAR1 and ADAR2 could be purified as RNA-independent homodimers from Sf9 cells (32). At the same time, O’Connell and coworkers found that the Drosophila ADAR (dADAR) dimerized in an RNA-dependent fashion and that this self-association was required for editing (33).

The fact that specific non-neural substrates have not been identified for either ADAR1 or ADAR2, despite the ubiquitous expression profile of these enzymes in all tissue types, coupled with a possible RNA dependence of their dimerization complicates study of ADAR self-association. We therefore decided to create fusion proteins of both ADAR family members with cyan and yellow fluorescent protein (CFP and YFP) and probe their association by fluorescence resonance energy transfer (FRET) in human cells (34). Using this approach, we have been able to observe both homo- and heterodimerization of ADAR1 and ADAR2 in HeLa cells. The results of RNase treatment before FRET measurement suggest that homodimerization of ADAR1 and ADAR2 as well as heterodimerization are independent of RNA binding.

EXPERIMENTAL PROCEDURES

Mammalian expression constructs—Human ADAR1 p110 was amplified by PCR from pJEL/hADAR1/H6 using primers containing HindIII and BamHI restriction sites. Human ADAR2 was PCR amplified from a previously described template (31) using primers containing EcoRI and SalI restriction sites. ADAR1 and ADAR2 were then inserted into pEYFP-C1, pEYFP-N1, pECFP-C1, and pECFP-N1 (Clonetech) using the appropriate restriction enzymes. Plasmids were transformed into chemically competent DH5α E. coli, amplified, and purified using a plasmid mini-prep kit (Sigma).

Cell Culture and Transfection—HeLa cells were cultured as monolayers in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% FBS, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C and 5% CO2. Cells (2 x 105) were plated on 25 mm coverslips (Fisher Scientific) in six-well tissue culture dishes and were allowed to adhere for 24 hours. The cells were transiently transfected with 1-5 µg plasmid DNA using Perfectin (Gene Therapy Systems) according to the manufacturer’s protocol and analyzed 20-24 hours post-transfection. In order to examine the RNA dependence of localization and ADAR association, HeLa cells transfected with 1-5 µg plasmid DNA were permeabilized in Gal-Screen Buffer B (Applied Biosystems) for 10 minutes. They were then washed twice with PBS before being treated with an RNase cocktail (RNase A: 200 µg/µl; RNase T1: 100 U/µl; RNase V1: 0.5 U/µl) for 30 minutes.

Fluorescence Microscopy—Transiently transfected cells on coverslips were rinsed 3 times in PBS and
FRET Analysis of ADAR Dimerization

fixed for 15 minutes in freshly prepared 4% formaldehyde (Sigma) at room temperature. Coverslips were washed a further 3 times in PBS before being mounted onto slides using Vectashield with DAPI (Vector Laboratories). Images were collected with a Zeiss Laser Scanning Confocal Microscope (LSM 510 NLO Meat) mounted on a Zeiss Axiovert 200M inverted microscope with a 40X F-fluar lens (N.A. 1.3) equipped with 4 visible lasers with 5 laser lines and a spectral Meta detector. The 458 and 514 nm laser lines (emitted from a 25 mW argon laser) were used to image CFP and YFP. Band pass filters of 462-484 nm and 580-612 nm were used in collecting emission from CFP and YFP respectively.

Fluorescence Resonance Energy Transfer—FRET experiments were performed on fixed cells using the donor recovery after acceptor photobleach method (34). First, images were obtained in the CFP and YFP channels and the intensities of the signals were calculated. YFP was then selectively photobleached at 514 nm in a defined region of the cell. A second set of images was then obtained using the same conditions as prior to photobleaching. FRET efficiency was calculated as follows:

$$\text{FRET efficiency} = \frac{(D_{\text{post}} - B_{\text{post}}) - (D_{\text{pre}} - B_{\text{pre}})}{(D_{\text{pre}} - B_{\text{pre}})}$$

where D is the donor channel intensity, B is the background intensity, and "pre" and "post" indicate measurements before and after photobleaching. A non-bleached area of the same cell served as an internal control.

RESULTS

Cellular Localization of ADAR1 and ADAR2 Fluorescent Fusion Proteins—In order to examine both cellular localization and associations of ADAR1 and ADAR2 by fluorescence, we cloned the cDNA for human ADAR1 and ADAR2 into the mammalian expression vectors ECFP and EYFP to produce ADAR fusion proteins with N-terminal CFP and YFP under the control of a CMV promoter. We also prepared expression constructs with CFP C-terminal to the deaminase sequence in order to be able to probe for directionality in any observed association.

Previous studies have revealed a dynamic association of ADAR1 and ADAR2 with the nucleolus; ADAR2 shuttles between the nucleolus and the nucleoplasm while ADAR1 shuttles between the nucleolus, nucleoplasm, and cytoplasm (an interferon inducible form of ADAR1 is cytoplasmic; 35-37). We determined the localization of the N-terminal CFP ADAR fusion proteins in transfected HeLa cells by confocal fluorescence microscopy observing, as expected, a predominantly nucleolar localization for these proteins (Fig. 1). Identical results were achieved using either C-terminal CFP or N-terminal YFP expression systems (data not shown).

Homo and Heterodimerization of ADAR1 and ADAR2—Having established the nucleolar localization of the fluorescently tagged ADAR1 and ADAR2, we performed co-transfections of HeLa cells using expression vectors for N-terminal CFP/YFP ADAR1 together and CFP/YFP ADAR2 together as well as control transfections with plasmids expressing CFP and YFP alone. A FRET experiment in this system involves the direct or indirect measurement, using fluorescence, of energy transfer between CFP and YFP following specific excitation of the CFP fluorophore. In order to simplify analysis, FRET signals were quantified by measurement of donor recovery following photobleaching. Briefly, CFP fluorescence was measured at 475 nm, following excitation at 458 nm with a laser, both before and after specific photobleaching of YFP. The increase in CFP fluorescence after YFP photobleaching corresponds to the original amount of energy transferred from CFP to YFP; thus the difference in CFP fluorescence prior to and after photobleaching corresponds to the level of FRET. Using this method, we were not able to measure any FRET between CFP and YFP alone. However, when N-terminal CFP ADAR1 was excited in the presence of N-terminal YFP ADAR1, we measured a FRET efficiency, between the fusion proteins localized to the nucleolus, of 18% (Fig. 2A, B; Table 1). For comparison, in a system expressing tandem CFP•YFP joined by a short ten amino acid linker, FRET efficiencies of 35% were measured (data not shown). When we analyzed cells transfected with N-terminal CFP ADAR1 and N-terminal YFP ADAR2, we were able to measure FRET efficiencies of 19% within the nucleolus (Fig. 2C, D; Table 1). Thus, as assayed by
FRET, both ADAR1 and ADAR2 form homodimers in the nucleolus.

We repeated co-transfection experiments with vectors expressing N-terminal CFP ADAR1 or ADAR2 as well as C-terminal YFP ADAR1 or ADAR2. Because the FRET signal varies with r^6 (38) we reasoned that measurements made with the fluorophores in different orientations might yield information on the disposition of the ADAR monomers with respect to one another. However, FRET levels measured using these expression constructs were essentially the same as observed in our initial experiment (data not shown). Thus, the FRET experiment does not reveal the orientation of individual ADAR proteins with respect to one another in the dimer.

After determining that ADAR1 and ADAR2 form homodimers within the cell, we wanted to examine the possibility of heterodimerization between isoforms. We performed co-transfections in HeLa cells using N-terminal CFP ADAR2 and N-terminal YFP ADAR1. Both isoforms localized to identical nucleoli within the cells, and we were again able to measure a FRET signal between the two nucleolar localized fusion proteins (Fig. 3; Table 1). The amount of FRET observed between ADAR1 and ADAR2 was 12% which is consistent with the formation of ADAR heterodimers in the nucleolus. Again, no difference in FRET signal was measured when co-transfecting N-terminal CFP ADAR2 and C-terminal YFP ADAR1; therefore an orientation of dimerization was impossible to determine.

RNA Binding and ADAR Dimerization—One of the reasons for examining ADAR dimerization in living cells is the possibility of a dependence on RNA or other proteins for dimerization: in vitro studies of ADAR association are in disagreement in this regard (31-33). Indeed, studies of ADAR dimerization are complicated by the fact that the endogenous RNA substrates for ADAR1 and for ADAR2 in most cells are unknown. The results of our FRET experiments suggest that ADAR1 and ADAR2 form both homo- and heterodimers in the nucleolus — we also wished to examine whether disruption of RNA binding by ADAR1 or ADAR2 affected cellular localization and formation of homo- or heterodimers. Emeson and coworkers previously noted that ADAR2 localization to the nucleolus was dependent on rRNA synthesis and was also abolished upon RNase treatment of cells (35). We therefore decided to examine the RNA dependence of ADAR self-association by RNase treatment of cells expressing the fluorescent fusion proteins.

We repeated transient transfections, as described above, to yield cells expressing combinations of CFP/YFP ADAR1 and CFP/YFP ADAR2. Transfected cells were permeablized and treated with a cocktail of RNases before being examined to determine the cellular localization of the fusion proteins. Consistent with earlier studies (35), permeablization of the cells did not affect ADAR localization but subsequent treatment with RNase abolished the exclusive nucleolar localization of both fusion proteins which were now observed throughout the nucleus and to a small extent within the cytoplasm (Fig. 4A, B; data not shown). In the case of RNase treated cells co-expressing CFP ADAR2 and YFP ADAR1, although exclusive nucleolar localization was abolished, the proteins remained co-localized (Fig. 4C).

We next performed a series of FRET experiments on RNase treated cells in which we measured donor recovery following photobleaching of YFP in extra-nucleolar regions of the cell. FRET efficiencies similar to those observed in untreated cells were measured for ADAR1, ADAR2, and ADAR1/ADAR2 expressing cells (Fig. 4; Table 1). These results suggest that neither homo- nor heterodimerization of ADAR1/ADAR2 is dependent on RNA binding.

A recent paper by Bass and coworkers reports the crystal structure of the deaminase domain of ADAR2 (39) and suggests that dimerization is not mediated through this domain. We expressed CFP and YFP fusion proteins of the same deaminase construct (ADAR2 299-701) used in the structural studies in HeLa cells, determined their localization, and looked for evidence of FRET between the fluorophores. The deaminase fusion proteins did not localize to the nucleolus — instead showing a diffuse expression throughout the cell — and did not undergo FRET (data not shown). This supports the conclusion that dimerization is not mediated by the deaminase domain and previous suggestions from work with Drosophila ADAR that ADAR self-association is mediated by N-terminal regions of the protein (33).
DISCUSSION

We have examined the localization and association of human ADAR1 and ADAR2 in HeLa cell culture by transient expression of fluorescent fusion proteins followed by confocal fluorescence microscopy. Our observations are in agreement with the previously reported primary localization of both ADAR1 and ADAR2 to the nucleolus. The FRET studies reported here also demonstrate that nucleolar ADAR1 and ADAR2 form both homo- and heterodimers. The observation of ADAR homodimerization is consistent with previous in vitro studies (31-33); heterodimerization has not been previously observed. In order to examine the RNA dependence of ADAR localization and dimerization, we treated cells expressing the fluorescent fusions with RNase; this had the effect of abolishing the exclusive nucleolar localization of both proteins but did not affect FRET between them. Thus, as observed by Emeson and colleagues (35), nucleolar localization is dependent on RNA binding. However the observation of FRET in the RNase treated cells also strongly suggests that ADAR dimerization is independent of RNA binding.

Two aspects of the results reported here are especially relevant to previous reports of ADAR dimerization. In our original report of ADAR2 self-association (31), we suggested that dimerization was RNA-dependent, a result supported by the observations of Gallo et al. (33) with respect to dADAR. This was contradicted by Nishikura and coworkers in their examination of ADAR1 and ADAR2 homodimers: in this study, extensive RNase treatment did not disrupt the isolated homodimers (32). The experiments reported here also support a model of RNA-independent dimerization. How can these two contrasting conclusions be reconciled? With respect to the results of the experiments with dADAR we would note that the RNA-dependence of dimerization was principally based on the examination of two A to E mutations in the first dsRNA binding domain of the protein (dsRBD). An examination of high-resolution crystal structures of dsRBDs (40, 41) shows that the residues in question are predicted to be located on the hydrophobic interior of the domain. Thus mutation of these amino acids to a polar, charged residue would be expected to significantly destabilize the domain structure and suggests that these mutants are not ideal for studying ADAR dimerization. Our own initial conclusions on the RNA-dependence of dimerization were based on the observation of an RNA-dependent ADAR2-ADAR2 crosslink as well as the sequential formation of monomer and dimer bound RNA complexes as assayed by native PAGE (31). In light of the results reported here, it is likely that the crosslink represents an RNA-dependent rearrangement upon dimer binding to RNA rather than evidence of dimerization per se. Bass and coworkers have reported evidence for an RNA dependent rearrangement of ADAR2 involving N-terminal residues of the protein (42). The observation of two distinct ADAR-RNA complexes by native PAGE would then reflect a more complex model than previously suggested involving a concentration dependent ADAR monomer/dimer equilibrium in solution.

The observation of ADAR heterodimers in vivo contrasts with the studies of Nishikura and coworkers in which only homodimers were isolated (32). This suggests that association of the heterodimer is weaker than the homodimer or that heterodimer formation/stability involves higher order complexes with other factors and that these were disrupted upon purification under the relatively high salt conditions (400 mM) used in that study.

What is the evidence that ADAR complexes observed in the nucleolus are functionally relevant? The fact that the dsRNA structures which contain the best characterized cellular editing sites are formed by base-pairing between exon and intron sequences means that editing precedes splicing. When the editing competent portion of the GluR-B pre-mRNA was expressed in HeLa cells, this transcript accumulated in the nucleoplasm but not the nucleolus and also appeared to redirect localization of ADAR2 and ADAR1 to non-nucleolar sites containing the GluR-B transcript (43). On the other hand, there are several pieces of evidence which suggest that RNA editing, of at least certain RNAs may take place in the nucleolus. In a recent study, Cavaillé and coworkers targeted specific editing substrates either to the nucleoplasm or nucleolus by placing their transcription under the control of a Pol II or Pol I promoter respectively and cotransfecting the corresponding minigenes with ADAR1 or ADAR2 expressing vectors (44). Interestingly, the RNAs localized to the nucleolus were efficiently edited only at sites targeted by ADAR2 suggesting that,
for these transcripts, ADAR2, but not ADAR1, is active in the nucleolus. A second, intriguing piece of evidence which may link editing to the nucleolus is the discovery in mice of a brain specific C/D small nucleolar RNA (snoRNA), MBII-52, which is predicted to target one of the A to I editing sites of the 5-HT2C receptor pre-RNA for 2'-O-methylation (44), a modification which has been shown to decrease editing efficiency. Thus it is possible that transient nucleolar localization of the 5-HT2C RNA modulates editing.

We have shown that in the cell ADAR family members associate through the formation of both homo- and heterodimers and that this association is RNA independent. Previous in vitro mixing studies have indicated that combinations of ADAR family members can attenuate or modulate editing activity (45). These observations considered in light of the results reported here suggest that ADAR homo- and heterodimerization may be an important mechanism to regulate efficiency and specificity of editing in the cell.

REFERENCES


**FOOTNOTES**

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1. The abbreviations used are: ADAR, adenosine deaminase that acts on RNA; FRET, fluorescence resonance energy transfer.

**FIGURE LEGENDS**

**Figure 1. Localization of ADAR1 and ADAR2 Fluorescent Fusion Proteins.** **A.** Confocal fluorescence imaging of HeLa cells transfected with CFP•ADAR1 (left) showing DAPI imaging of the nucleus (middle). A merged image (right) shows CFP•ADAR1 localizing to regions of faint DAPI fluorescence which correspond to nucleoli. **B.** Confocal fluorescence imaging of HeLa cells transfected with CFP•ADAR2 (left) showing DAPI imaging of the nucleus (middle). A merged image (right) shows CFP•ADAR2 localizing to regions of faint DAPI fluorescence which correspond to nucleoli. Bar = 5 µm.

**Figure 2. Homodimerization of ADAR1 and ADAR2.** **A.** Confocal fluorescence imaging of HeLa cells co-transfected with CFP•ADAR1 and YFP•ADAR1. Shown are: emissions filtered at 462-484 nm (top; CFP), emissions filtered at 580-612 nm (middle; YFP), and merge (bottom). Images were taken before (left) and after (right) specifically photobleaching YFP within a nucleolus of the cell. Bar = 5 µm. **B.** Quantification of images before and after photobleaching (indicated by arrow). CFP fluorescence is shown for the photobleached nucleolus (open circles) and a non-bleached nucleolus (closed circles). The fluorescence of YFP is shown for the same photobleached nucleolus (open squares) and non-bleached nucleolus (closed squares). Quantifications corresponding to images at left are indicated (◆, ◆). **C.**
Experiment as in panel A but for cells co-transfected with CFP•ADAR2 and YFP•ADAR2.  
P. Quantification as in panel B for images shown in panel C.

Figure 3. Heterodimerization of ADAR1 and ADAR2.  
A. Confocal images obtained from HeLa cells co-transfected with CFP•ADAR2 (top) and YFP•ADAR1 (middle).  A merged image of the two top images is shown in the lower panels.  Images were taken before (left) and after (right) specifically photobleaching YFP within a nucleolus of the cell.  Bar = 5 µm.  
B. Quantification of images before and after photobleaching (indicated by arrow).  CFP fluorescence is shown for the photobleached nucleolus (open circles) and a non-bleached nucleolus (closed circles).  The fluorescence of YFP is shown for the same photobleached nucleolus (open squares) and non-bleached nucleolus (closed squares).  Quantifications corresponding to images at left are indicated (◆, ◆).

Figure 4. RNA Independent Homo- and Heterodimerization of ADAR Family Members.  
A. Confocal fluorescence imaging of HeLa cells co-transfected with CFP•ADAR1 and YFP•ADAR1 and then treated with RNase.  Shown are:  emissions filtered at 462-484 nm (top;  CFP), emissions filtered at 580-612 nm (middle;  YFP), and merge (bottom).  Images were taken before (left) and after (right) specifically photobleaching YFP within a region of the cell nucleus.  Bar = 5 µm.  
B. Quantification of images before and after photobleaching (indicated by arrow).  CFP fluorescence is shown for the photobleached area (open circles) and a non-bleached area (closed circles).  The fluorescence of YFP is shown for the same photobleached area (open squares) and non-bleached area (closed squares).  Quantifications corresponding to images at left are indicated (◆, ◆).  
C. Experiment as in panel A but for cells co-transfected with CFP•ADAR2 and YFP•ADAR2.  
D. Quantification as in panel B for images shown in panel C.  
E. Experiment as in panel A but for cells co-transfected with CFP•ADAR2 and YFP•ADAR1.  
F. Quantification as in panel B for images shown in panel E.
Table 1  % CFP•ADAR-YFP•ADAR FRET in HeLa Cells

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*a %FRET values are average of five measurements; reported errors are one standard deviation from the calculated value.
Figure 3
Figure 4
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