Requirement of Dimerization for RNA Editing Activity of Adenosine Deaminases Acting on RNA*

Dan-Sung C. Cho‡,‡, Weidong Yang‡, Joshua T. Lee, Ramin Shiekhattar, John M. Murray‡, and Kazuko Nishikura**

From The Wistar Institute, Philadelphia, Pennsylvania 19104 and the Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Adenosine deaminases acting on RNA (ADAR) convert adenosine residues into inosines in double-stranded RNA. Three vertebrate ADAR gene family members, ADAR1, ADAR2, and ADAR3, have been identified. The catalytic domain of all three ADAR gene family members is very similar to that of Escherichia coli cytidine deaminase and APOBEC-1. Homodimerization is essential for the enzyme activity of those cytidine deaminases. In this study, we investigated the formation of complexes between differentially epitope-tagged ADAR monomers by sequential affinity chromatography and size exclusion column chromatography. Both ADAR1 and ADAR2 form a stable enzymatically active homodimer complex, whereas ADAR3 remains as a monomeric, enzymatically inactive form. No heterodimer complex formation among different ADAR gene family members was detected. Analysis of HeLa and mouse brain nuclear extracts suggested that endogenous ADAR1 and ADAR2 both form a homodimer complex. Interestingly, endogenous ADAR3 also appears to form a homodimer complex, indicating the presence of a brain-specific mechanism for ADAR3 dimerization. Homodimer formation may be necessary for ADAR to act as active deaminases. Analysis of dimer complexes consisting of one wild-type and one mutant monomer suggests functional interactions between the two subunits during site-selective RNA editing.

One type of RNA editing converts adenosine residues into inosine within the double-stranded RNA (dsRNA) region of substrate RNAs (1–3). Because inosine is treated as guanosine by the translational machinery, this A-to-I editing could lead to functional alterations of the affected genes. For instance, A-to-I RNA editing results in the expression of editing isoforms of glutamate receptor (GluR) ion channel subunits (4, 5) and serotonin 2C subtype receptors (5-HT2C) (6). Editing of the so-called “Q/R” site of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid Glur-B subunit dramatically decreases the Ca2+ permeability of the channel (7). Substantial reduction in G-protein coupling efficiency is noted with A-to-I editing of 5-HT2C RNA at five positions (A to E sites) located in the intracellular II loop region (6, 8–10). A-to-I RNA editing also occurs in non-coding sequences. Editing of its own intron sequence by adenosine deaminase acting on RNA (ADAR) 2 creates an alternative splice acceptor site leading to synthesis of a truncated translation product, which may be a negative feedback mechanism to regulate the activity of ADAR2 (11). In all these examples, a dsRNA structure formed between the exonic sequences containing an editing site(s) and downstream or upstream intronic sequences has been proven to be essential for editing (4–6, 12). Systematic search with a recently devised method for cloning of inosine-containing RNAs has led to identification of more than two dozen editing sites occurring in the intron and 3′-untranslated regions of new target genes. A-to-I RNA editing of these non-coding regions may affect the splicing rate, the translation efficiency, or stability of the edited mRNAs (13). Furthermore, the intronic and untranslated region sequences subjected to A-to-I RNA editing often contain common repetitive elements such as Alu and LINE1 repeats forming a long dsRNA structure, raising the possibility that A-to-I RNA editing may be involved in a mechanism regulating the very abundant repetitive sequences in mammalian genomes (2, 3, 13). Finally, A-to-I RNA editing of dsRNAs derived from transgenes appears to prevent silencing of the transgenes regulated by RNA interference, revealing the potential intersection of the two mechanisms, RNA editing and RNA interference both evolved to deal with dsRNA (14).

Members of the ADAR gene family have been implicated as the enzymes responsible for A-to-I RNA editing. Three separate mammalian gene family members (ADAR1 to ADAR3) have been identified (15–22). Data base search has identified corresponding fish ADARs revealing the conservation of the complete set of ADAR gene family members in vertebrates through evolution (23, 24). In invertebrates, a single Drosophila dADAR, very similar to mammalian ADAR2 (25), and two less conserved Caenorhabditis elegans c.e.ADAR1 and c.e.ADAR2 have been identified (15, 26). Mammalian ADAR1 and ADAR2 are detected ubiquitously (15, 16, 18–20), whereas the expression of mammalian ADAR3, Drosophila dADAR, and C. elegans c.e.ADAR1 is restricted mainly to nervous systems (21, 22, 25, 27). Analysis of ADAR null mutation phenotypes has revealed the importance of A-to-I RNA editing. Flies with a null mutation of dADAR, although viable, display defective locomotion and behavior accompanied by various neurological and ana-
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A longstanding question with regard to the enzymatic activities of ADARs is whether they act as monomeric or oligomeric forms and whether oligomerization plays a role in the site-selective editing mechanism. The catalytic domain of ADAR is very similar to that of the cytidine deaminase gene family (1, 2, 15). E. coli cytidine deaminase forms a homodimer (34), and does APOBEC-1, another cytidine deaminase involved in C-to-U editing in mammalian cells, is made as a dimer at the N terminus region (39). The region contains a Kozak sequence that is strongly preferred by baculovirus for protein translation initiation at the N terminus region (39). The region amplified by PCR was confirmed by sequencing. Baculovirus expression constructs were then transformed into DH10Bac for transposition into the bacmid and subjected to blue/white screening for identification of recombinant baculoviruses.

Expression of the ADAR Recombinant Baculovirus—Sf9 cells were grown to a density of 2 x 10^6 cells/ml and infected with either a single or a combination of two ADAR recombinant viruses (1:1 ratio) at a multiplicity of infection of 10–20. At 48 h post-infection, 1 x 10^6 cells were collected.

Extract Preparation—All procedures were carried out at 4 °C. HeLa cell extract was prepared as described previously (40). Mouse brain nuclear extract was prepared by the Dignam method (41) with a minor modification as follows. Fresh mouse brains were minced using a pair of scissors, and further homogenized by a motor-driven Potter homogenizer in 3 times the packed cell volume of phosphate-buffered saline. The cell pellet was suspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 1 x complete protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN), and 0.5 mM phenylmethylsulfonyl fluoride, and kept on ice for 20 min. Cells were lysed by 10–20 strokes with a glass Dounce homogenizer followed by centrifugation at 10,000 rpm for 15 min in a Type 65 Ti Beckman rotor. The nuclear pellet was suspended in 3 pellet volumes of a buffer containing 20 mM HEPES (pH 7.9), 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 1.0 mM DTT, 1 x complete protease inhibitor mixture, and 0.5 mM phenylmethylsulfonyl fluoride. After five gentle strokes in a glass Dounce homogenizer, the protein extract was collected by centrifugation at 30,000 rpm for 30 min.

Sequential Epitope Tag Affinity Column Purification of ADAR Dimer Complexes—All column chromatography procedures were carried out at 4 °C. Total cell extract was prepared from Sf9 cells infected with a single or a combination of two recombinant baculoviruses (38). The cell extract, dialyzed against buffer A (0.05 mM Tris, pH 7.0, 0.15 mM NaCl, 5 mM KCl, 1.0 mM DTT, 0.025 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40) was first passed through a 1.0-mL anti-FLAG M2-monoclonal antibody (mAb)-agarose gel (Sigma) affinity column equilibrated with buffer A containing 0.15 mM NaCl and 1 mM β-mercaptoethanol instead of 1 mM DTT. After washing the column with 10 mL each of buffer A containing 0.15 mM NaCl and 1 mM β-mercaptoethanol, the complex was eluted with buffer containing 0.15 mM NaCl and 200 μg/mL FLAG peptide. The pooled peak fractions were dialyzed against buffer B (10 mM Tris, pH 7.5, 0.3 mM NaCl, 20% glycerol, 0.05% Nonidet P-40, 1 mM β-mercaptoethanol) and then applied to a TALON metal resin (BD Biosciences, Palo Alto, CA) affinity column. Following extensive washing with buffer B containing 10 mM imidazole, proteins were eluted by 10 mM imidazole. The yield of recombinant proteins during the sequential affinity chromatography was followed by Western blotting analysis using an anti-FLAG M2 mAb (Sigma) or anti-His6 6XH mAb (BD Biosciences). The purity of recombinant proteins purified by the first 17094

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Size Exclusion Column Chromatography Analysis—Purified ADAR proteins (1 µg) or crude nuclear extract (2 mg) were applied to a 24-ml (1 x 30 cm) column of Superose 12 HR 10/30 (Amersham Biosciences) for size exclusion chromatography. The buffer system used was 0.05 M Tris (pH 7.0), 0.5 M NaCl, 5 mM EDTA, 1 mM DTT, 20% glycerol, and 0.1% Nonidet P-40. Purified recombinant ADAR proteins were concentrated to 100 µl using Centricon (Amicon) before applying to the column. Fractions (0.5 ml) were collected at a flow rate of 0.4 ml/min using a fast protein liquid chromatography system. The molecular weight of ADAR (monomer or oligomer) was estimated by comparison with molecular weight standards obtained from Sigma; bovine thyroglobulin (669,000), horse spleen apoferritin (443,000), sweet potato β-amylase (200,000), yeast alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and bovine carbonic anhydrase (29,000). The peak for the ADAR complex was confirmed by Western blotting analysis, and the peak position of the marker proteins was determined by measuring the optical absorption at 280 nm.

In Vitro RNA Editing Assay—Editing of a synthetic 5-HT_{2C} RNA C5 was assayed in vitro as described previously (22), using 1 x or 2 x purified recombinant homodimer complexes as well as 2 x purified heterodimer complexes consisting of one wild-type and another non-functional mutant ADAR monomer. The standard editing reaction contained 20 fmol of a synthetic C5 RNA substrate, 10 ng of recombinant actinomycin, 0.02 M Hepes (pH 7.0), 0.1 M NaCl, 10% glycerol, 5 mM EDTA, 1 mM DTT, and 250 units/ml RNasin (Promega). The reactions were incubated at 30°C for various times. Quantitation of editing efficiency at five sites of 5-HT_{2C} RNA was carried out by dideoxyribonucleotide primer extension assay as described previously (10, 22). The ratio of the edited and unedited RNAs was estimated by quantifying the radioactivity of the primer-extended products with a phosphor-imaging system (Amersham Biosciences).

Western Immunoblot Analysis—Proteins were fractionated on an SDS-8% polyacrylamide gel and transferred to Immobilon™-P nylon membrane (Millipore, Bedford, MA). Blots were blocked in a buffer consisting of phosphate-buffered saline and 3% nonfat dry milk. Mabs 15.8.6, 1.3.1, and 3.591 were detected by antibody conjugated goat antibodies directed against mouse immunoglobulins (Kirkegaard and Perry Lab., Gaithersburg, MD) and chemiluminescence staining using Renaissance™ (PerkinElmer Life Sciences).

RESULTS

Recombinant ADAR1 and ADAR2 but Not ADAR3 Proteins Form Stable Homodimeric Complexes—A set of baculovirus constructs for ectopic expression of ADAR1, ADAR2, and ADAR3 with either a FLAG or a 6His epitope tag at the N terminus were prepared. Two different sizes of ADAR1 protein, a full-length 150 kDa and a shorter 110 kDa form (p150 and p110) are synthesized because of differential usage of two Met initiation codons (17). The full-length ADAR1 (p150), and ADAR2a among the four known splicing isoforms of ADAR2 (19, 20), were investigated in the present studies. FLAG- and 6His epitope-tagged ADAR1, ADAR2, or ADAR3 proteins were coexpressed in SF9 cells infected with approximately a 1:1 ratio of two different recombinant baculoviruses, and purified by sequential affinity chromatography, first on M2 anti-FLAG mAb-agarose gel and then TALON metal resin as schematically shown in Fig. 1. Each purification step was monitored by Western analysis using anti-FLAG or anti-6His antibody. Both ADAR1 and ADAR2 were purified as oligomeric complexes containing both FLAG- and 6His-tagged protein (Fig. 2, lanes 3 and 8 for ADAR1 and lanes 4 and 9 for ADAR2). The binding of FLAG-tagged ADAR1 or ADAR2 protein to the first affinity column was nearly complete, whereas a substantial amount (30 to 50%) of the 6His-tagged ADAR1 or ADAR2 protein was detected in this first flow-through fraction as expected. The unbound 6His-tagged ADAR protein represents, most likely, the oligomeric complex consisting of 6His-tagged monomers only (see Fig. 1). In contrast, only FLAG-tagged ADAR (30 to 50% of the total FLAG-tagged protein present in the original extracts, again representing complexes composed entirely of FLAG-tagged monomers) but almost no 6His-tagged ADAR was detected in the flow-through of the second TALON affinity column, indicating complete binding of the 6His-tagged ADAR that had been preselected by FLAG mAb-agarose gel chromatography (i.e. the oligomeric complex consisting of both FLAG- and 6His-tagged monomers). Overall, the yield of the 2 x purified oligomeric complex was 30 to 50% of the ADAR present in the extracts, consistent with the amount expected on the basis of monomers sorting randomly into oligomeric complexes without regard to the FLAG or 6His tag. This essentially complete recovery establishes that the oligomer represents the major form of the complex, and also that our sequential affinity column purification scheme did not selectively enrich a rare form of the complex. The copurification of FLAG with 6His epitope-tagged ADAR1 as well as ADAR2 were confirmed through a similar sequential affinity column chromatography, but in the reverse order, i.e. TALON metal resin first and then M2 anti-FLAG mAb-agarose gel (data not shown). In contrast, recombinant ADAR3 protein was detected in Western analysis only by using the antibody corresponding to the type of affinity chromatography applied first (Fig. 2, lane 5) but not by the reciprocal antibody corresponding to the second affinity chromatography (Fig. 2, lane 10).
results clearly indicate that recombinant ADAR1 and ADAR2 but not ADAR3 form oligomers.

The apparent molecular mass of ADAR1 (full-length p150 form), ADAR2a, and ADAR3 have been estimated to be 150, 90, and 80 kDa, respectively, by SDS-PAGE (18–22, 30, 31). To determine the size(s) of ADAR1 and ADAR2 recombinant proteins purified through sequential affinity chromatography, the oligomeric complexes eluted from the second affinity column were fractionated on a Superose 12 size exclusion column (Fig. 3, A and B, top panels). Based on the standard size markers, the sizes of ADAR1 and ADAR2 oligomeric forms were estimated to be 300 and 180 kDa, respectively, indicating that they are both homodimers.

The sequential affinity column chromatography purification procedure as designed precludes detection of ADAR monomer in the 2× purified fraction (Fig. 1). Therefore, to check for free monomers we also examined the apparent sizes of ADAR1 and ADAR2 when expressed as single epitope-tagged proteins and purified by a single affinity column (1× purified). We found that the fractionation profiles for both 1× purified ADAR1 and ADAR2 were identical to those of the 2× purified ADAR proteins. Distinctive elution peaks anticipated for the monomeric forms of ADAR1 (150 kDa) and ADAR2 (90 kDa) were not detected (data not shown). In addition, we carried out the sequential affinity chromatography of two proteins, each separately tagged with FLAG and 6His epitope and purified on a single affinity column (1× purified) following in vitro mixing and incubation. We found that there was no significant in vitro exchange of two differentially epitope-tagged ADAR1 or ADAR2 proteins, at least during a 2-h incubation at 30 °C (data not shown). Taken together, our results suggest that both ADAR1 and ADAR2 recombinant proteins ectopically expressed in Sf9 cells form predominantly a stable homodimeric complex.

Superose 12 column chromatography conducted with FLAG epitope-tagged recombinant ADAR3 proteins purified by a single affinity column chromatography (1× purified) revealed a complex elution pattern significantly different from one expected for its monomeric state (Fig. 3C, upper panel). Because the silver staining of the recombinant ADAR3 proteins indicated that they were more than 90% homogeneous, the results were surprising. Although the presence of a minor peak at the position expected for the monomer form was clearly detected (80 kDa, indicated by an arrow), the majority of recombinant ADAR3 proteins eluted as a broad smear covering a range from ~400 kDa to much smaller than the monomeric form, suggesting possible nonspecific interaction with the Superose 12 matrix. Using high salt (2 M NaCl) and different pH buffers did not change the elution pattern.

We previously have reported the presence of a ssRNA binding domain located within the arginine-rich R domain (22). Thus, we reasoned that binding of RNA molecules from the insect cells might be responsible for the unusual migration of ADAR3. However, size fractionation of the ADAR3 after treating extensively with RNases specific for both ssRNA and dsRNA (see below) was identical to that of untreated protein (data not shown). In conclusion, the results of sequential affinity column purification strongly suggest a monomeric state for recombinant ADAR3, but we currently do not understand the reason for its unusual migration on Superose 12.

Interestingly, FLAG epitope-tagged ADAR1 and ADAR2 expressed in Sf9 cells and purified on M2 anti-FLAG mAb-agarose gels have been demonstrated to be enzymatically active in deamination of adenosines on a long synthetic dsRNA substrate or in the site-selective in vitro RNA editing of GluR or 5-HT2c-R substrate RNAs (20, 30), whereas recombinant ADAR3 proteins are inactive in these assays (22). We now realize that the ADAR1 and ADAR2 recombinant proteins used for our previous studies were predominantly homodimeric forms, and thus it may be that the enzymatic activity of these two recombinant ADARs are related to a dimer formation.

**RNA Independent Homodimerization of ADAR1 and ADAR2—**

The size exclusion chromatography with doubly purified fractions revealed no large oligomeric complexes that would form via binding of multiple ADAR monomers to a long dsRNA. However, homodimerization of ADAR1 and ADAR2 recombinant protein could be dependent on the presence of a short dsRNA substrate of Sf9 cell origin serving to bridge two monomers of ADAR1 or ADAR2. Both RNA-dependent and independent homodimerization of the dsRNA-activated protein kinase PKR has been reported (45–45). The overall arrangement of functional domains in PKR, two dsRNA binding domains at the N terminus, and a separate catalytic domain, is
somewhat similar to ADAR. We therefore tested the homodimer complex copurified by sequential affinity chromatography for their sensitivity to single-strand and double-strand specific ribonuclease treatments (Fig. 4). FLAG and 6His epitope-tagged ADAR1 or ADAR2 recombinant proteins remained together as a dimer regardless of RNase A and T1 (ssRNA specific) or RNase V1 (dsRNA specific) treatment. Thus, the association of two different epitope-tagged monomers is unlikely to be mediated through an RNA molecule(s) (Fig. 4B). On the other hand, it is still possible that an RNA molecule directly involved in formation of the ADAR homodimer may be resistant to the ribonuclease digestion because of its close contact with ADAR proteins. To eliminate the possibility, we looked for RNA molecules bound to the ADAR homodimer complex by $^{32}$P labeling and PAGE analysis of the labeled RNAs. Approximately 2 μg of 2× purified ADAR1 or ADAR2 was subjected to proteinase K digestion and subsequent RNA extraction. The total RNA extracted was then labeled using $^{32}$P-labeled pCP and T4 RNA ligase and analyzed by 7 M urea-PAGE. By including known amounts of a synthetic 21-nucleotide RNA molecule as an internal control, we concluded that no significant level of RNA is present (less than 0.1 ng of RNA for 2 μg of the doubly purified ADAR homodimer). This is less than one RNA base per dimer, clearly insufficient to act as a bridge between monomeric proteins (data not shown).

Homodimerization of Native ADARs—To confirm homodimerization of native ADAR1 and ADAR2, HeLa cell and mouse brain nuclear extracts were subjected to Superose 12 size exclusion column chromatography. In HeLa cells, both p150 and p110 forms of ADAR1 were detected (Fig. 3A, middle panel). The peak of native ADAR1 p150 coincided with that of the recombinant p150 homodimer complex (300 kDa), and ADAR1 p110 proteins also eluted in the fractions expected for its homodimer complex (220 kDa). In mouse brain, only the p110 form of ADAR1 was detected in nuclear extracts, and it appeared in the fractions expected for the homodimer (Fig. 3A, lower panel). Native ADAR2 proteins appear also to exist mainly as a complex of homodimer size (Fig. 3B, middle and lower panels). Both the ADAR2a and ADAR2b splicing isoforms of native human ADAR2 differing in size by 40 amino acid residues (19, 20) were detected in the HeLa nuclear extract. In mouse, the size difference, only 10 amino acid residues, was not sufficient for detection of these two isoforms separately (18). We noted a slightly broader elution peak of native ADAR1 and ADAR2 especially with a tailing toward smaller molecular weight regions in comparison with recombinant homodimer complexes, possibly indicating the presence of some monomer form (Fig. 3, A and B, middle and lower panels). Both the ADAR2a and ADAR2b splicing isoforms of native ADAR2 differing in size by 40 amino acid residues (19, 20) were detected in the HeLa nuclear extract. In mouse, the size difference, only 10 amino acid residues, was not sufficient for detection of these two isoforms separately (18). We noted a slightly broader elution peak of native ADAR1 and ADAR2 especially with a tailing toward smaller molecular weight regions in comparison with recombinant homodimer complexes, possibly indicating the presence of some monomer form (Fig. 3, A and B, middle and lower panels). As with the recombinant protein, fractionation of the native mouse brain ADAR3 resulted in a complex elution pattern, but with a minor peak that coincides with the size for the anticipated ADAR3 homodimer (160 kDa), suggesting the possibility that native ADAR3 may also form a homodimeric complex in brain (Fig. 3C, lower panel). Unlike the recombinant protein, smearing

ADAR1 (panel A), ADAR2 (panel B), or ADAR3 (panel C). Recombinant ADAR1 (A, upper panel) and ADAR2 (B, upper panel) proteins, differentially epitope-tagged and purified by sequential affinity column chromatography (2X), and recombinant ADAR3 proteins (C, upper panel) purified by a single M2 FLAG mAb affinity column (1X), were analyzed. Extracts made from HeLa cells or mouse brain were also investigated. The positions of size marker proteins are indicated by open arrowheads. Expected positions of the ADAR1 homodimer (300 kDa for p150 and 220 kDa for p110) and the ADAR2 homodimer (180 kDa) are indicated by arrows (panels A and B). Positions of ADAR3 monomer (80 kDa) and homodimer (160 kDa) are also indicated by arrows (panel C). Recombinant ADAR3 proteins were detected also in all fractions collected beyond the 30 fractions shown, up to fraction 50.

**Fig. 3.** Analysis of ADAR oligomeric complexes by Superose 12 gel filtration column chromatography. Oligomeric forms of ADAR proteins fractionated by Superose 12 gel filtration column chromatography were analyzed by Western blotting analysis using mAb specific to
into fractions corresponding to small molecules was not observed with native ADAR3. More importantly, no obvious peak of monomer was detected for native ADAR3 (Fig. 3C, lower panel), in contrast to recombinant ADAR3 (Fig. 3C, upper panel). It should be pointed that a fraction of native ADAR1 (detected for certain extract preparations but not seen clearly in Fig. 3A, middle and lower panels) and ADAR3 proteins (Fig. 3C, lower panel) migrated with an apparent molecular weight of >600,000, whereas no larger complex of native ADAR2 was detected other than the homodimer size complex in the extracts of HeLa cells and mouse brain. Interestingly, digestion of the extracts with RNase A and T1 prior to Superose 12 size exclusion chromatography resulted in shifting of at least a part of the larger ADAR1 or ADAR3 complexes to complexes of homodimer size. Most importantly, however, RNase digestion (ssRNA and dsRNA specific) did not affect the size of the homodimer-like complex of ADAR1, ADAR2, or ADAR3, indicating RNA independent homodimerization of native ADARs (data not shown). We have recently reported the association of both ADAR1 and ADAR2 with large nuclear ribonucleoprotein particles, consisting of four splicesomal subunits that assemble together with the pre-mRNA. However, the size of the complexes observed in the present studies are far smaller than the large nuclear RNP particles (200 S), which can be detected with the HeLa nuclear extract prepared through a specific gentle extraction procedure in the presence of 2 mM vanadyl ribonucleoside RNase inhibitor (42). Thus, the nature of the larger complexes of native ADAR1 and ADAR3 detected in the present studies (both RNase digestion sensitive and resistant) is not clear at this time.

**Different Members of the ADAR Gene Family Do Not Associate as Heterodimers**—We next investigated the possibility of heterodimer complex formation between two different members of the ADAR gene family (Fig. 5). Two differentially epitope-tagged ADAR gene family members, i.e. ADAR1 and ADAR2 (lanes 1 and 7), ADAR1 and ADAR3 (lanes 2 and 8), or ADAR2 and ADAR3 (lanes 3 and 9), were coexpressed in the same cell (Sf9) and purified by sequential affinity chromatography. The presence of both ADAR proteins tagged with FLAG
shown that site-directed mutagenesis of Glu912 of ADAR1 hydrolytic deamination reaction (15, 38). We have previously a critical role in proton transfer functions required for the nase gene family members. These residues are believed to play family members as well as cytidine and deoxycytidylate deami-
monomers. The glutamate residues Glu 912 of ADAR1 and

HAE

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12

ent ADAR gene family members was detected (Fig. 6)

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ent ADAR gene family members (data not shown). These results suggest that formation of heteromeric complexes among different ADAR gene family members is unlikely.

Functional Interaction between the Two Monomers of the ADAR Homodimer—The two monomers within an ADAR1 or ADAR2 homodimer may bind to two separate substrate RNAs and deaminate adenosine residues at each catalytic center independently. Alternatively, the two monomers may act co-

opatively for conversion of an adenosine residue of a single substrate RNA. To obtain some insights into possible func-
tional interaction between the two monomers, we examined the enzymatic activities of a heterodimer complex made between wild-type and a non-functional ADAR mutant in comparison to those of mutant or wild-type homodimers (Fig. 6). ADAR1 E912A with a Glu912 → Ala substitution and ADAR2a E396A with a Glu906 → Ala substitution were used as the mutant monomers. The glutamate residues Glu912 of ADAR1 and Glu906 of ADAR2 are located within the tripeptide sequences HAE and PCG, which are highly conserved among ADAR gene family members as well as cytidine and deoxycytidylate deami-
nase gene family members. These residues are believed to play a critical role in proton transfer functions required for the hydrolytic deamination reaction (15, 38). We have previously shown that site-directed mutagenesis of Glu912 of ADAR1 (E912A) results in complete abolishment of the deaminase activity without affecting substrate RNA binding capability (38). Formation of the heterodimer between one wild-type and one mutant monomer was first confirmed by their copurification through sequential affinity column chromatography as above (data not shown). Non-selective ADAR activity, which converts multiple adenosines to inosines in a sequence inde-

pended manner, was determined on a long 575-bp synthetic c-myc dsRNA (Fig. 6A). Site selective A-to-I RNA editing activity was monitored by determining the editing of 5-HT$_{2C}$R RNA at the A site by ADAR1 and the D site by ADAR2. Preferential editing of the A and D sites by ADAR1 and ADAR2, respectively, has been demonstrated previously in vitro using recombinant proteins (6, 22, 46). Preliminary time course experi-
ments were conducted separately to choose conditions under which the enzymatic reaction remains first-order in enzyme concentration, so that the results can be compared quantita-
tively. The enzymatic activities of heterodimers consisting of one wild-type and one non-functional mutant monomer were found to be approximately half (55% for ADAR1 and 52% for ADAR2) of the wild-type homodimer activity when tested with the long c-myc dsRNA substrate (Fig. 6A). In contrast, site-

selective RNA editing activity by the heterodimer, determined on 5-HT$_{2C}$R RNA, decreased to ~30% of the wild-type ho-

modimer activity (Fig. 6B). These results may indicate that natural substrate RNAs induce cooperative interactions be-

between the two monomers in the wild-type homodimer complex. Presumably, the three-dimensional structure of natural sub-

strates such as 5-HT$_{2C}$R RNA, which includes short dsRNA regions, loops, and bulges, facilitate simultaneous interactions with both monomers in the dimer complex.

DISCUSSION

Homodimerization of ADAR1 and ADAR2—In this study, we have demonstrated that recombinant ADAR1 and ADAR2 both exist predominantly as stable homodimers. Purification of the complexes via sequential affinity chromatography with two

![Fig. 6. Functional interaction between two subunits of the dimer complex in A-to-I RNA editing.](http://www.jbc.org)
different epitope tags revealed for the first time the presence of oligomeric complexes, whereas size exclusion column chromatography identified the complexes as homodimers. The homodimer formation is mediated by protein-protein interaction between two monomers and is independent of binding to RNA. Although we originally assumed that both oligomeric as well as monomeric forms of ADAR proteins might be present in equilibrium, our results suggest that recombinant ADAR1 or ADAR2 ectopically expressed in S99 cells appears to form predominantly a homodimer, possibly immediately after translation or even during translation as occurs for some dimeric proteins (e.g. tubulin). Furthermore, homodimers once formed appear to be very stable without detectable exchange of their monomer components under physiological conditions.

Native ADAR1 biochemically purified from various sources (bovine liver, calf thymus, and Xenopus oocytes) has been reported to have various sizes (80 to 120 kDa), smaller than the full-length p150 form, probably because of translation initiation at the internal methionine codon (equivalent to our p110 form) or truncation of the N terminus as a result of nonspecific proteolysis (47–49). In contrast to our findings in this study with recombinant ADAR1, biochemically purified native ADAR1 was found to be a monomer by size exclusion column chromatography and by glycerol gradient sedimentation analysis (47–49). In addition to the differences in protein size and possible species differences, another possible explanation for the discrepancy is that native ADAR1, predominantly in the homodimer state, might become dissociated into monomers because of the rather vigorous biochemical purification procedures applied. Interestingly, it has been reported that the monomeric form of Xenopus ADAR1, once biochemically purified, does not re-dimerize even under low ionic strength conditions (49). In all previous studies, the purification of native ADAR1 was assayed by enzymatic activity, suggesting that the biochemically purified monomeric ADAR1 was capable of deaminating the dsRNA substrate used in their assay (46–48).

An alternative interpretation consistent with that data is that ADAR1 protein dissociated into monomers because of the nonphysiological conditions applied during purification, but was reconstituted into homodimers upon binding to dsRNA during the A-to-I base modification assay, which restored its enzymatic activity. Recently, homodimerization of ADAR2 on a dsRNA substrate has been proposed based on the results of kinetic analysis (50). A-to-I RNA editing was observed only under conditions allowing ternary complex formation between the ADAR2 homodimer and GluR-B RNA substrate, indicating a requirement for ADAR2 dimer formation for its site-selective editing activity (50). Binding of a substrate RNA and acceleration of homodimerization of PKR has been reported also (43–45).

Our results on recombinant ADAR1 and ADAR2 are supported by size exclusion column chromatography analysis of HeLa and mouse brain nuclear extracts, which detected the anticipated homodimer size complex of native ADAR1 and ADAR2. The native complexes were detected by Western blotting analysis of the crude extracts using ADAR1- or ADAR2-specific antibodies, and thus we cannot exclude the possibility that they represent the monomer associated with some currently unknown molecule (protein or RNA). However, the identical sizes of the native complexes and recombinant homodimers suggest that a large fraction of native ADAR1 or ADAR2 most likely also exist as homodimers.

**Possible Dimerization of Native ADAR3 in Brain**—In contrast to the results with ADAR1 and ADAR2, we could not detect the homodimer of recombinant ADAR3. Recombinant ADAR3 ectopically expressed in S99 cells apparently remains as a monomer, which may explain its lack of enzymatic activity (22). The behavior of the recombinant ADAR3 during size exclusion column chromatography was also aberrant, eluting from a Superose 12 column as a broad smear, including fractions corresponding to physically impossible sizes. It may be that a majority of recombinant ADAR3, incapable of homodimerizing, does not have a uniform structure and migrates through the column without forming a distinctive fractionation peak. Alternatively, monomeric recombinant ADAR3 may interact non-specifically with the matrix material of the sizing column used, possibly because of lack of a required post-translational modification that takes place in brain but not in S99 insect cells. This hypothesis then predicts that native ADAR3 protein may form a homodimer and behave differently from the recombinant proteins during size exclusion column chromatography. Indeed, size fractionation analysis of mouse brain extracts on a Superose 12 column revealed an elution peak corresponding to a complex of ~160 kDa, the anticipated size of the ADAR3 homodimer, in addition to a separate peak corresponding to a much larger molecular mass complex (>600 kDa). The size of the 160-kDa complex also corresponds to the size of a potential ADAR2/ADAR3 heterodimer (Fig. 3C, lower panel).

Heterodimerization among proteins related to the human ADAR family has been observed in other organisms. The ADAT gene family (tRNA-specific A-to-I editing enzymes) has been identified in yeast because of their deaminase domain sequence homology to ADAR (1). ADAT1 edits A37 of tRNAAla as a monomer (51), whereas A34 at the first anticodon position of tRNAAla is edited specifically by a heterodimer formed by ADAT2 and ADAT3 (52). ADAT2 is the catalytically active subunit, whereas ADAT3 is the regulatory subunit not directly involved in the A-to-I deamination mechanism (52). More recently, the possibility of heterodimer formation between c.e.ADAR1 and c.e.ADAR2 has also been indicated (27). We were intrigued by the possibility that enzymatically inactive ADAR3 might form a heterodimer complex with an enzymatically active ADAR member (e.g. ADAR2), and therefore investigated possible heterodimer formation among three different ADARs. We found no indication of any heterodimer formation between two different ADAR gene family members, including the heterodimer consisting of ADAR2 and ADAR3, at least among recombinant proteins co-expressed in the same S99 insect cell. Taken together with our preliminary results from coimmunoprecipitation experiments of mouse brain extracts, we conclude that the complex eluting around 160 kDa is likely to represent a ADAR3 homodimer that forms only in mouse brain.

**Interaction of Two Monomers**—The structural basis of the interactions between two monomer subunits of ADAR1 or ADAR2 is currently unknown. The deaminase domain structure of ADAR is predicted to have a similarity to *E. coli* cytidine deaminase (1, 15). In the x-ray crystal structure of *E. coli* cytidine deaminase the homodimer has a 2-fold symmetry axis, indicating that the two monomers are structurally indistinguishable and predicted to be functionally equivalent (34). Molecular modeling of another cytidine deaminase APOBEC-1, ApoB RNA editing enzyme, predicts a structural configuration of the homodimer very similar to that of *E. coli* cytidine deaminase (37). Extensive interactions widely spread over many regions are involved in the formation of the monomer-monomer interface of *E. coli* cytidine deaminase and APOBEC-1 homodimers (34, 37). The active site in each monomer is completed only with contributions from the other partner subunit (34, 37). We have recently conducted studies to map the regions required for formation of the homodimer using the ADAR1
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mutant baculovirus constructs (38) coexpressed with the wild-type construct and sequential affinity chromatography purification of the dimer complex. Although the conclusions of those studies remain preliminary because of the unstable nature of certain deletion constructs, it appears that the interface interactions of the two monomers occurs over a widespread region including the deaminase domain as well as the dsRNA binding domains. In contrast, the N-terminal region containing the Z-DNA binding domain (amino acids 1 to 295) is not required, because formation of the heterodimer between p150 and p110 of ADAR1 can be detected. The regions critical for nuclear import or export of ADAR1 have been mapped recently (53, 54). It would be interesting to know if formation of the homodimer is involved also in the nuclear-cytoplasmic shuttling mechanism. The stable nature of the recombinant ADAR1 and ADAR2 homodimers predict its de novo formation during translation and transport as a homodimer unit. However, it is also possible that the monomer is used as a transport form (nuclear import or export), whereas homodimer formation may be a part of a mechanism to concentrate the active complex in one compartment of the cell.

Dimerization is known to affect the enzymatic activity as well as substrate specificity (45, 52). Each dsRNA binding domain of ADAR is independently capable of binding to a dsRNA region as short as 15 to 20 bp. A number of ADAR proteins bind to multiple sites of a long completely complementary dsRNA (55), but also to a discrete site of a specific hairpin RNA (56). Thus, each monomer of ADAR1 or ADAR2 may bind to a separate dsRNA molecule through its own dsRNA binding domains. However, it is also possible that one homodimer binds to a single substrate RNA whereas the dsRNA binding domains of the two monomers make cooperative interactions. In an attempt to understand the functional interactions of the two monomers, enzymatic activity of a heterodimer consisting of a pair of ADAR monomers or each monomer with a substrate RNA. A model for this enzyme may be independent bound and deaminated by each monomer. In contrast, a substrate RNA with relatively short dsRNA regions, loops, and bulges such as 5′-H5p′R may be bound by the dsRNA binding domains of both monomers, and deaminated after formation of an active complex in which interactions between the two monomers brings about alignment of a select adenosine residue with one of the two catalytic centers. Our future studies will define interactions of the two subunits of ADAR1 and ADAR2 homodimers and address their significance for enzymatic activity as well as intracellular localization.

Acknowledgments—We thank C.-X. Chen for preparation of epitope-tagged ADAR expression constructs and the Wistar Genomics/Microarray, Expression Vector-Recombinant Protein Production, and Hybridoma facilities for excellent technical assistance. We also thank the Wistar editorial services department for preparing the manuscript.

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D-S. C. Cho and K. Nishikura, unpublished results.
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Requirement of Dimerization for RNA Editing Activity of Adenosine Deaminases Acting on RNA
Dan-Sung C. Cho, Weidong Yang, Joshua T. Lee, Ramin Shiekhattar, John M. Murray and Kazuko Nishikura

J. Biol. Chem. 2003, 278:17093-17102.
doi: 10.1074/jbc.M213127200 originally published online March 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M213127200

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