THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

An extra double-stranded RNA binding domain confers high activity to a squid RNA editing enzyme

An extra double-stranded RNA binding domain confers high activity to a squid RNA editing enzyme

JUAN PABLO PALAVICINI,[1] MARY A. O'CONNELL,[2] and JOSHUA J.C. ROSENTHAL[1,3]

[1]Institute of Neurobiology, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico 00901, USA
[2]MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, United Kingdom
[3]Department of Biochemistry, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico 00936, USA

ABSTRACT

RNA editing by adenosine deamination is particularly prevalent in the squid nervous system. We hypothesized that the squid editing enzyme might contain structural differences that help explain this phenomenon. As a first step, a squid adenosine deaminase that acts on RNA (sqADAR2a) cDNA and the gene that encodes it were cloned from the giant axon system. PCR and RNase protection assays showed that a splice variant of this clone (sqADAR2b) was also expressed in this tissue. Both versions are homologous to the vertebrate ADAR2 family. sqADAR2b encodes a conventional ADAR2 family member with an evolutionarily conserved deaminase domain and two double-stranded RNA binding domains (dsRBD). sqADAR2a differs from sqADAR2b by containing an optional exon that encodes an "extra" dsRBD. Both splice variants are expressed at comparable levels and are extensively edited, each in a unique pattern. Recombinant sqADAR2a and sqADAR2b, produced in *Pichia pastoris*, are both active on duplex RNA. Using a standard 48-h protein induction, both sqADAR2a and sqADAR2b exhibit promiscuous self-editing; however, this activity is particularly robust for sqADAR2a. By decreasing the induction time to 16 h, self-editing was mostly eliminated. We next tested the ability of sqADAR2a and sqADAR2b to edit two K^+ channel mRNAs in vitro. Both substrates are known to be edited in squid. For each mRNA, sqADAR2a edited many more sites than sqADAR2b. These data suggest that the "extra" dsRBD confers high activity on sqADAR2a.

Keywords: ADAR; RNA editing; squid; dsRBD; potassium channels; *Pichia pastoris*

INTRODUCTION

Over the past decade, genome sequencing projects have shown that an organism's complexity is not proportional to the number of genes that it possesses. Accordingly, other mechanisms must be important to generate genetic diversity. RNA editing is one example. Among higher metazoans (Bilateria) adenosine deamination (A-to-I) is the most common form of RNA editing; however, the extent to which it is used to change codons varies greatly. In vertebrates, it regulates the nervous system function in important ways. For example, calcium permeability at the synapse is regulated by editing GluR-B subunit pre-mRNAs of ionotrophic glutamate receptors (Kohler et al. 1993). In addition, G-protein signaling can be regulated by editing pre-mRNAs for the serotonin receptor 5-HT$_{2C}$ (Burns et al.

1997). Finally, repetitive firing can be modulated by editing mRNAs for the potassium channel Kv1.1A (Bhalla et al. 2004). Knockout of either of the two mammalian editing enzymes is lethal (Higuchi et al. 2000; Wang et al. 2000, 2004; Hartner et al. 2004). Although A-to-I editing clearly has important consequences in vertebrates, available data would suggest that it is infrequently used to modify protein structure. In spite of numerous mammalian genome sequences, EST and SNP databases, fewer than 30 mRNAs have been found to be edited in their coding sequence, at an average of ~1.8 sites per transcript (Clutterbuck et al. 2005; Levanon et al. 2005; Ohlson et al. 2005; Gommans et al. 2008).

Recoding by A-to-I editing appears to be much more extensive in higher invertebrates (Protostomes). For example, in *Drosophila* comparative genomics and bioinformatic screens have so far uncovered 55 targets, each edited at an average of ~2.2 sites per transcript (Hoopengardner et al. 2003; Xia et al. 2005; Stapleton et al. 2006). As with vertebrates, most of these sites are present in mRNAs that code for proteins involved in nervous system function. Although not lethal, deletion of the single *Drosophila* editing enzyme

causes severe locomotion defects (Keegan et al. 2005). In squid a surprisingly large number of editing sites have been uncovered with comparably little effort. Because no cephalopod genome sequences or EST libraries are available, bioinformatic approaches have not been used. However, simply by comparing individual cDNAs to gene sequences, 48 sites have been uncovered in the only three targets that have been examined. In mRNAs for a K_v2 potassium channel expressed in the brain of *Loligo pealei* 18 editing sites were identified over a small 360-nucleotide (nt) region encoding the channel's pore (Patton et al. 1997). Further, in mRNAs for a K_v1 potassium channel expressed in the giant axon of *Loligo opalescens*, 16 sites were identified (Rosenthal and Bezanilla 1902). Recent data on mRNAs for a second K_v1 potassium channel from the giant axon in *Loligo pealei* shows 14 editing sites (C. Colina, J. Monterrubio, and J. Rosenthal, unpubl.). Some of these sites cause substantial effects on channel function. For example, the edit R87G in sqKv1.1A mRNA interferes with channel tetramerization. Finally, unpublished data from our laboratory, suggest that editing is not limited to K^+ channel mRNAs but also occurs in transcripts for Na^+ channels, ion pumps, and ion exchangers.

Why is A-to-I editing so extensive in squid? In order to approach this question, it is useful to consider the deamination reaction. In all in vitro systems studied to date, the biochemistry of editing is relatively simple, requiring just an enzyme and a RNA substrate. The target adenosine is usually embedded in an imperfect duplex structure. The editing enzyme (ADAR) recognizes this double-stranded structure and catalyzes the deamination. It is reasonable to speculate that squid have either evolved more secondary structures in their mRNAs that are suitable for editing or they have modified their ADAR to increase its activity. We find the latter more probable.

A garden variety ADAR is composed of two basic domains: a variable number of double-stranded RNA binding domains (dsRBDs) and a highly conserved deaminase domain. Structurally similar dsRBDs are found in a wide variety of RNA binding proteins (Doyle and Jantsch 2002; Chang and Ramos 2005). The ADAR deaminase domain, on the other hand, has a unusual structure: it is folded around a central inositol hexakisphosphate (IP_6) molecule (Macbeth et al. 2005). It is thought that by binding to the secondary structure surrounding an editing site, the dsRBDs guide the enzyme to a specific adenosine and the deaminase domain carries out the catalytic reaction. Vertebrates have two ADAR genes that encode enzymes with demonstrable catalytic activities: ADAR1 and ADAR2. A major structural difference between these is that ADAR1 has three dsRBDS and ADAR2 has only two. Each recognizes a distinct but overlapping sets of substrates (Melcher et al. 1996). Invertebrate ADARs have been cloned from *Drosophila* and *Caenorhabditis elegans*. The single *Drosophila* ADAR is more similar to the mammalian ADAR2 family

(Palladino et al. 2000). *C. elegans* has two ADAR genes, neither of which can be classified as ADAR1 or ADAR2 (Tonkin et al. 2002; Keegan et al. 2004). Here we report that squid express an ADAR2 homolog, with an ''extra'' dsRBD like ADAR1s, and this feature confers an increased level of activity.

RESULTS

Isolation of two ADAR homologs from squid

Previous studies have shown extensive editing in giant axon transcripts (Rosenthal and Bezanilla 1902; Patton et al. 1997), so we concentrated our efforts to isolate a squid ADAR on this tissue. As a first step, we used degenerate PCR primers to highly conserved residues in the deaminase domain to amplify a 398-nt fragment that was homologous to mammalian ADAR2s. This cDNA clone was then used as a hybridization probe to screen a cDNA library made from the stellate ganglion of *Loligo opalescens* (Rosenthal and Gilly 1993; Rosenthal et al. 1996). The cell bodies of the giant axon are located in the giant fiber lobe (GFL), which is a part of, but not all of, the stellate ganglion. Therefore, this library represents the giant axon system and other neurons. Out of a screen of $\sim10^6$ clones, a full-length ADAR homolog was isolated (sqADAR2a). This clone was sequenced to completion. To verify that it is expressed in the giant axon system, we designed PCR primers over its start and stop codons and amplified the full-length sequence from RNA isolated exclusively from the GFL (Fig. 1A). Although this reaction produced a robust band at the expected size (~2.4 kb), it also consistently yielded a smaller band (~2.1 kb). The smaller product (sqADAR2b), which was cloned and sequenced, encodes an apparent splice variant.

sqADAR2a and sqADAR2b were named to reflect their similarity to the vertebrate ADAR2 family (see Discussion) (Bass et al. 1997). sqADAR2a differs from sqADAR2b by a 297-nt insertion after nucleotide 42 (Fig. 1B). We verified that the two are splice variants by cloning the *sqADAR2* gene by PCR from *L. opalescens* genomic DNA. The *sqADAR2* gene is composed of four constitutive exons and one optional exon. There are two introns, the first of which has only been partially cloned. The second is 925 nt. The optional exon, which distinguishes the isoforms, is significant because it encodes a 99 amino acid insertion that includes a dsRBD (Fig. 2). Thus, the major difference between the splice variants is that sqADAR2a encodes an unusual ADAR2 variant with three dsRBDs, and sqADAR2b encodes a conventional ADAR2 variant with just two. Considering the distance between squid and man, sqADAR2 exhibits a remarkable degree of similarity to human ADAR2. For example, the dsRBDs share $\sim80\%$ identity and the deaminase domains share 61% identity. Four residues critical for catalyzing the hydrolytic deamination
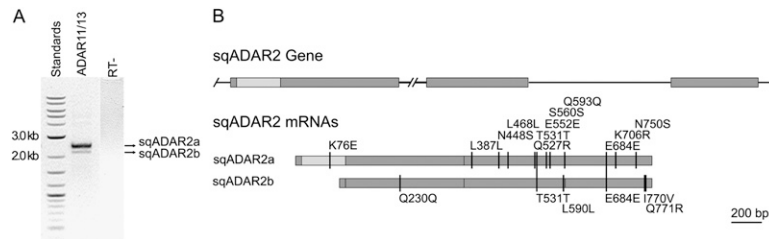
FIGURE 1. Two splice variants of the *sqADAR2* gene in the giant axon system. (*A*) PCR amplification of full-length sqADAR2a, with primers *before* and *after* the start and stop codons (ADAR11/13), yields two products: sqADAR2a (~2.4 kb) and sqADAR2b (~2.1 kb). RNA for cDNA was isolated from the GFL. The positions of DNA standards are indicated on the *left*. A no reverse transcriptase control (RT−) reaction was also included. (*B*) Schematic representation of squid ADAR gene, the two mRNA splice variants, and the positions of edited codons within these mRNAs. Constitutive and optional exons are represented by dark and light gray boxes, respectively. Introns are represented by lines. A break in the line for the first constitutive intron indicates that its length and sequence have not been determined. Gray lines indicate the positions of editing sites. The codon change caused by the editing event is written *above* or *below* each line (the first letter indicates the genomically encoded amino acid, the number indicates the codon position within sqADAR2a, and the second letter indicates the edited codon). Gray lines that extend through both sqADAR2a and sqADAR2b indicated that both splice variants are edited at these positions.

reaction in ADARs and cytidine deaminases are also conserved in squid (Betts et al. 1994; Kim et al. 1994; Lai et al. 1995; Macbeth et al. 2005). In sqADAR2 these residues are H458, C516, and C580, which coordinate the zinc ion, and E460, which shuttles the proton from water. Further, the crystal structure of hADAR's catalytic domain shows 24 positions that coordinate IP_6, 22 of which are conserved in sqADAR2 (Macbeth et al. 2005). Based on these features, the squid clones bear all the hallmarks of ADAR.

Both sqADAR2 isoforms are extensively edited in squid

When cloning and sequencing the sqADAR2a and sqADAR2b cDNAs, it became apparent that there was extensive A or G variation at specific positions. To investigate whether these were RNA editing sites, we sequenced 50 individual clones of each variant and compared them to the gene sequence. At multiple sites where the gene sequence contained an A, the cDNA sequence contained an A or a G. In total, 12 editing sites were identified for sqADAR2a and 6 for sqADAR2b, two of which were shared (Fig. 1B). Of these sites, five cause amino acid changes for sqADAR2a, and two for sqADAR2b. All recoding sites are located in the enzyme's deaminase domain, except for K76E, which is in the sqADAR2a's "extra" dsRBD. Clearly, these editing sites may cause substantial ADAR diversity in the giant axon system, and they are the subject of ongoing investigation in our laboratory. However, the rest of this paper focuses on the importance of the "extra" dsRBD of sqADAR2a. Accordingly, all constructs used in this study encode the genomic (unedited) versions of *sqADAR2a* and *sqADAR2b*.

Expression of squid ADARs in the giant axon system

With an "extra" dsRBD, sqADAR2a has a unique domain structure. How extensively is this version expressed in the squid nervous system? The PCR shown in Figure 1A suggests that both versions are expressed in the giant axon system. In order to verify sqADAR2a/b expression in the GFL, and to estimate their relative abundances, we used an RNase protection assay. Our strategy is outlined in Figure 3A. As a hybridization probe we used a 501-nt antisense RNA, 387 nt of which are complimentary to sqADAR2a and include a portion of the sequence encoding the "extra" dsRBD (the rest of the sequence is vector derived). Only 290 nt of this probe are complimentary to sqADAR2b, which lacks the optional exon. As expected, when the probe is hybridized to full-length sqADAR2a or sqADAR2b RNA transcribed in vitro, bands of 387 and 290 nt are protected (Fig. 3B). Both bands are also present when using GFL total RNA. We also processed RNA isolated from the optic lobe, a region of the central nervous system, and the results were similar. In the GFL we estimate that the sqADAR2a makes up $36 \pm 3\%$ (SD, $n = 4$) of the total and in the optic lobe it makes up $21 \pm 1\%$ (SD, $n = 4$). For each expected band, there was also a relatively faint product that was slightly larger. Because this was true for the RNA controls as well, it is probable that this represents either secondary structure that is not completely denatured during electrophoresis or small portion of the probe that is not effectively protected during RNAse digestion. Although sqADAR2b is more abundantly expressed, sqADAR2a makes a significant contribution to the total ADAR mRNA.

After verifying that both forms of sqADAR2 are expressed in the squid nervous system, we next focused on whether the "extra" dsRBD that differentiates the two isoforms affects function. Our strategy was to use in vitro editing assays to test both enzymes' activity, first on nonspecific double-stranded RNA, and then on K^+ channel transcripts known to be edited in squid using a recombinant enzyme. To produce sqADAR2a and sqADAR2b, we chose *Pichia pastoris* because it has successfully been used to express ADARs from other species (O'Connell et al. 1998; Ring et al. 2004; Keegan et al. 2007). sqADARs were tagged with an N-terminal FLAG epitope and six histidines at the C terminus. After induction, *Pichia* were disrupted with a French Press and the recombinant proteins were purified by elution from a Ni^{2+}-NTA column, and a subsequent elution from an anti-FLAG affinity column (Fig. 4A). For sqADAR2a, two bands are present after the Ni^{2+}-NTA
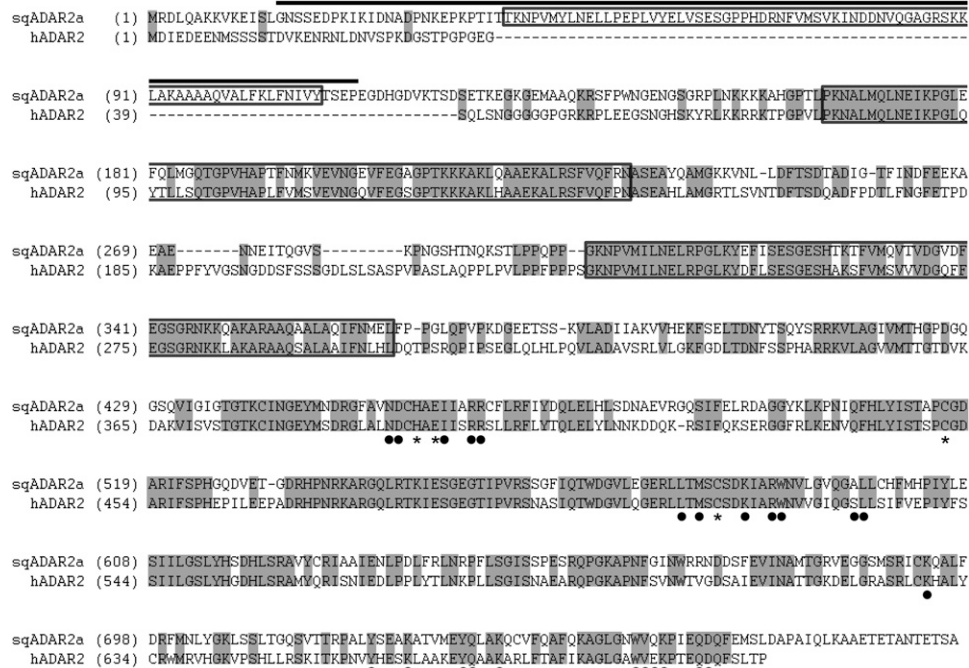
FIGURE 2. sqADAR2 is highly conserved. Amino acid sequence alignment of sqADAR2a (GenBank AN FJ478450) and human ADAR2 (hRED1-S, GenBank AN U82120). Open boxes indicate the positions of dsRBDs and the bold line indicates the position of the optional exon that encodes the "extra" dsRBD (the precise sequence that sqADAR2b [GenBank AN FJ478451] lacks). Gray boxes represent identity across both ADARs. Asterisks (*) refer to the three Zn^{2+}-chelating and proton-shuttling residues critical for deamination. The filled circles (•) refer to residues that hydrogen bond with IP6, either directly or through a water molecule (Macbeth et al. 2005).

elution, the ~89 kDa full-length product and a smaller one, perhaps due to an amino-terminal truncation. For both sqADAR2s, the FLAG elution yields a highly purified product as assessed by a protein gel stained with Coomassie.

Squid ADAR mRNAs encode active enzymes

Both sqADAR2a or sqADAR2b are functionally active (Fig. 4B). Further, over a range of protein concentrations, the activity of both enzymes appears similar and the end-state editing percentages are equivalent (Fig. 4C). In each case, after a 2-h incubation between 45% and 50% of the available adenosines are converted to inosines, similar to the enzymatic activity of other ADARs (Bass and Weintraub 1988; Palladino et al. 2000). Approximately 1.5 ng of recombinant ADAR produces half-maximum conversion. Thus, based on this data, there does not appear to be a major difference between the two sqADAR2s on nonspecific substrates. However, a more-extensive analysis of enzyme kinetics could uncover more subtle differences.

Squid ADARs edit their own messages in *Pichia*

Before assaying sqADAR2s on specific squid transcripts, we wanted to determine if the recombinant enzymes could edit their own mRNAs in *Pichia*, which would result in a heterogeneous protein preparation. For example, both human ADAR2 and *Drosophila* ADAR edit their own pre-mRNAs in vivo (Rueter et al. 1999; Palladino et al. 2000), and our results indicate that sqADARs are also edited in vivo. Moreover, *Drosophila* ADAR has been shown to self-edit in *Pichia* as well (Keegan et al. 2005). The established protocols for expressing ADAR in *Pichia* use a 48-h induction in order to maximize protein expression (O'Connell et al. 1998; Ring et al. 2004; Keegan et al. 2007). When we sequenced sqADAR2 cDNAs from cultures that had been induced for 48 h, we discovered extensive self-editing, particularly with sqADAR2a where over 90% of the clones had between one and five editing sites (Fig. 5A). With sqADAR2b, about 60% of the clones showed self-editing. In general, self-editing in sqADAR2b was less extensive than in sqADAR2a (although a very small percentage of sqADAR2b clones had between eight and 10 editing sites). In addition, these edits were scattered at multiple sites throughout the open reading frame. For example, sqADAR2a had 26 editing sites, each edited in between 6% and 34% of the clones. Seven of these sites are also edited in vivo (K76E, L387L, T531T, S560S, Q593Q, K706R, and N750S). sqADAR2b had 21 editing sites, each edited in between 6% and 24% of the clones. One of these sites is also edited in vivo (T531T).

These data were intriguing because they suggested that there might be a difference in enzymatic activity between the two splice variants. However, they also presented a
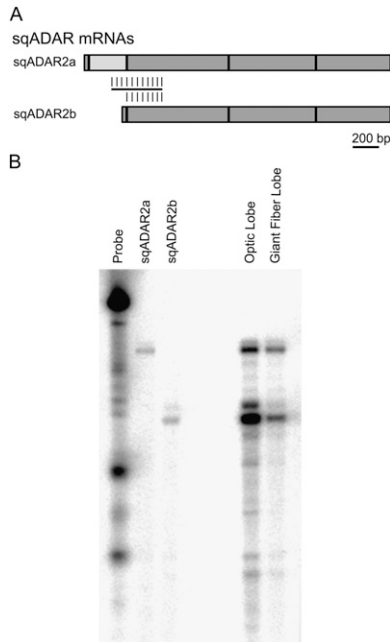
FIGURE 3. sqADAR2 splice variants are expressed at comparable levels. sqADAR2a and sqADAR2b expression levels were measured by RNase protection in two tissues: the OL and the GFL. Tissues were dissected from *L. opalescens*. (*A*) Schematic of probe homology for both sqADAR2a and sqADAR2a. The probe is represented by the bold horizontal line, and complementarity is denoted by vertical lines. Constitutive and optional exons are represented by dark and light gray boxes, respectively. (*B*) Phosphorimage of RNase protection assay. The probe is 501-nt long, and the sqADAR2a and sqADAR2a protected bands are 387 and 290 nt, respectively. Note that for each expected band, there is also a slightly larger product. This is true for both the positive controls and the experimental tissues, and is therefore probably an artifact.

problem: recombinant sqADAR2 produced after a 48-h induction would be heterogeneous. A previous study with *Drosophila* ADAR overcame this problem by changing the single edited codon to one that could not be edited by adenosine deamination (Keegan et al. 2005). However, with sqADAR2 in *Pichia* this approach would not be feasible because of the extensive number of codons that are edited.

To overcome this problem, we reasoned that self-editing would be less extensive after shorter induction times. Therefore, we tried to identify an induction time that produced sufficient protein expression but greatly reduced self-editing. After a 16-h induction, protein expression was reduced by ∼20-fold but there was still a sufficient yield for in vitro assays. More importantly, self-editing was dramatically reduced (Fig. 5B). For sqADAR2a 72% of the clones were unedited, and the vast majority of the remaining clones had a single editing event. These editing events occurred at four possible positions, three of which were silent. The one event that produced an amino acid change (K773R, at the extreme C terminus) was only found in 8% of the clones. For sqADAR2b, 92% of the clones were completely unedited and the remaining 8% were edited at

position K674R, the homologous position to K773R in sqADAR2a. We conclude that after a 16-h induction the population of recombinant sqADAR2a and sqADAR2a was sufficiently homogenous to initiate in vitro editing assays.

Squid ADARs edit giant axon K⁺ channels mRNAs in vitro

Having prepared largely homogenous proteins, we were now in a position to compare the ability of both isoforms to edit transcripts in vitro that are known to be edited in the giant axon system. We first selected sqKv1.1A, which is edited at 16 positions (Rosenthal and Bezanilla 1902; Rosenthal et al. 1996). Our strategy was to incubate full length, in vitro transcribed RNA with recombinant sqADAR2a or sqADAR2b, subsequently perform RT-PCR on the RNA, and then sequence individual clones. To simplify data collection and analysis, we concentrated on the first 700 nt, which can be analyzed in a single sequencing run and contain 13 out of the 16 in vivo editing sites. Table 1 summarizes the results from our assay. sqADAR2a edits in vitro transcribed sqKv1.1A at 6 sites with an efficiency between 6% and 44%. Surprisingly, sqADAR2b edited no sites, even though this enzyme was shown to be active on duplex RNA substrate. For sqADAR2a, two of the in vitro sites were at positions that are also edited in squid.

Although these results show a clear difference between the enzymatic activity of sqADAR2a and sqADAR2b on a specific substrate, we questioned why more of the in vivo editing sites were not edited in vitro as well. One possibility
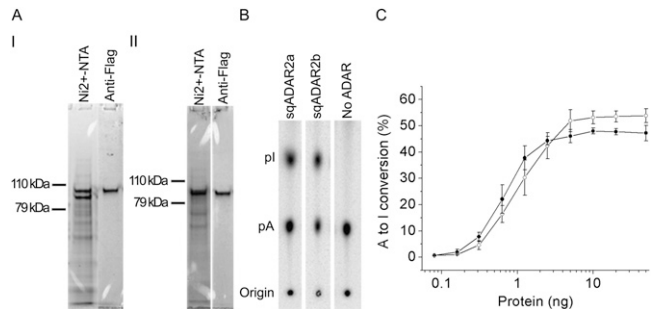

FIGURE 4. Recombinant sqADAR2a and sqADAR2b are both active. (*A*) SDS-PAGE gels of the purification of sqADAR2a (*I*) and sqADAR2b (*II*). Fractions eluted from Ni²⁺-NTA and α-FLAG columns were electrophoresed on a 8%–16% gradient gel and stained with Coomassie. The predicted size for sqADAR2a is ∼89 kDa and ∼78 kDa for sqADAR2b. Positions of protein standards are indicated on the *left*. (*B*) TLC analysis of nonspecific ADAR activity assay with radiolabeled, duplex RNA. Recombinant squid ADARs were incubated with radiolabeled dsRNA. Assays were then digested with P1 nuclease and then separated by thin-layer chromatography. The "No ADAR" lane was an equivalent incubation with no enzyme. (*C*) Various amounts of recombinant sqADAR2 protein (in ng: 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 10, 20, and 50) was incubated with dsRNA substrate containing 500 fmol total adenosine. Conversion percentage was calculated from PhosphorImager scans of the TLC plates.
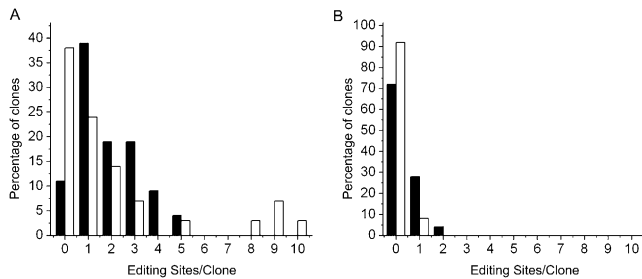
FIGURE 5. sqADAR2 is extensively self-edited in *Pichia pastoris*. Self-editing of individual mRNAs for sqADAR2a and sqADAR2b after 48 h (*A*) or 16 h (*B*) of induction. Filled bars represent sqADAR2a and open bars represent sqADAR2b. Forty-five to 55 individual clones were sequenced for each assay.

is that sqKv1.1A pre-mRNAs contain a single large intron between nucleotides 35 and 36. This sequence, which is not included in the in vitro transcribed RNA used for the assay, could be important for establishing the double-stranded structure that guides ADAR to edit some of these sites. Because our attempts to isolate this intron have been unsuccessful, we turned to a different K$^+$ channel transcript (Table 2). sqKv1.2A has the advantage of being intronless. As before, we focused on the first 700 nt, which contain seven out of 12 in vivo editing sites. As previously observed, sqADAR2a had a higher enzymatic activity than sqADAR2b, specifically editing 20 sites. In contrast to the sqKv1.1A transcript, sqADAR2b had enzymatic activity, editing three positions, all of which were also edited by sqADAR2a. Out of the seven in vivo sites, five were edited only by sqADAR2a, and one was edited by both enzymes (I171M). Interestingly, I171M was edited more efficiently by sqA-DAR2b. In vitro data from the self-editing sqKv1.1A and sqKv1.2A assays all support the hypothesis that sqADAR2a has a higher activity than sqADAR2b. In addition, by using sqKv1.2A, an intronless transcript, we were able to edit a greater percentage of the in vivo sites. However, there were still many positions only edited in the in vitro assay.

DISCUSSION

We originally hypothesized that the high editing activity observed in squid was due to the unique structure of its editing enzyme. Data in this paper support this idea. In general, squid ADAR is highly similar to vertebrate ADAR2, but it also exhibits an unusual feature: an "extra" dsRBD. Vertebrate ADAR1 family members also have three dsRBDs; however, the squid ADAR has all the hallmarks of an ADAR2-type protein (Fig. 6). First, when comparing the deaminase domain of squid ADAR with that of hADAR1 and hADAR2 (and hADAR3), squid ADAR is most similar to hADAR2 (Fig. 6A). A similar analysis comparing the dsRBDs yields a similar conclusion. Squid dsRBDs unambiguously group with those of hADAR2 and not with those of hADAR1 (Fig. 6B). For the sake of clarity,

we number the squid ADAR dsRBDs according to their order in sqADAR2a (i.e., dsRBD1–3). Our tree also shows that the second dsRBD from squid is most similar to the first from human, and the third from squid is homologous to the second from human. Interestingly, the first or "extra" dsRBD from squid is more similar to the squid's third than its second. This suggests that it probably arose through duplication of dsRBD3 (Fig. 6C).

Regardless of where it originated, the "extra" dsRBD in squid ADAR appears to have functional consequences. Both in vivo in our heterologous expression system and in vitro, sqADAR2a has higher activity than sqADAR2b. In *Pichia*, after 48 h of induction, almost all of sqADAR2a mRNAs were edited at one to five sites. More than half of the sqADAR2b mRNAs were edited as well, albeit less extensively. By decreasing the induction time, the extent of self-editing was greatly reduced; however, the overall pattern between sqADAR2a and sqADAR2b remained the same. These data indicate that when expressing ADARs in heterologous systems, it is critical to carefully assess self-editing. This is particularly true for sqADAR2a, but also for sqADAR2b as well. Self-editing has also been observed in *Drosophila* ADAR (Keegan et al. 2005), which like sqADAR2b has only two dsRBDs. It is therefore possible

TABLE 1. In vitro editing of sqKv1.1A cRNAs with recombinant sqADAR2s

Nt	Codon	In vitro	
		sqADAR2a	sqADAR2b
44	**D15G**	16	
48	**L16L**		
63	**E21E**		
103	**M35V**		
107	**Y36C**		
127	**I43V**		
134	**N45S**		
138	V46V	24	
139	**S47G**	**26**	
188	D63G	28	
190	T64A	44	
259	**R87G**		
271	S91G	6	
394	**K132E**		
418	**I140V**		
523	**M175V**		
535	**I179V**		

Editing percentages for specific editing sites identified in the first 700 bp of sqKv1.1A. In vitro results are from an assay where recombinant sqADAR2a or sqADAR2b were incubated with sqKv1.1A RNA. Codons that are edited in vivo, shown in bold, are based on Rosenthal and Bezanilla (2002) and on sequencing individual sqKv1.1A cDNA clones amplified from squid GFL RNA by RT-PCR. Results are based on 50 clones. Nt refers to the position of the edited adenosine. Codon refers to the specific codon affected by editing where the first letter is the unedited version and the second letter is the edited version.

TABLE 2. In vitro editing of sqKv1.2AA cRNAs with recombinant sqADAR2s

		In vitro	
Nt	Codon	sqADAR2a	sqADAR2b
13	K5E	14	
14	K5R	30	14
15	K5K	8	
17	K6R	6	
18	K6K	18	14
27	K9K	8	
32	K11R	16	
74	**K25R**		
75	**K25K**	**12**	
99	**T33T**	**14**	
110	Q37R	6	
127	N43D	12	
160	R54G	6	
284	Q95R	6	
289	**R97G**	**10**	
419	Y140C	6	
513	**I171M**	**8**	**22**
516	E172E	6	
639	**I213M**	**12**	
643	I215V	8	
666	**L222L**	**16**	

Editing percentages for specific editing sites identified in the first 700 bp of sqKv1.2A; same methods and conventions as for Table 1.

that vertebrate ADAR2s may also self-edit, even at sites that are not seen in vivo.

Our in vitro results largely corroborate those observed in *Pichia*. Combining the editing results from the two K$^+$ channel mRNAs known to be edited in vivo, sqADAR2a edited 26 sites while sqADAR2b only edited three. For sqKv1.2A, which is intronless, sqADAR2a could edit all the in vivo sites that were assayed, except for one (K25R). However, the editing activity was not entirely specific, as 14 sites were not observed in vivo. Of these, half were in a cluster at the 5′-end, occurring in the first 11 codons. The other half were scattered throughout the rest of the sequence that we analyzed. We do not believe that non-specific editing is due to excess sqADAR2 protein in the in vitro assay because K25R is edited in vivo but not in vitro.

These data also suggest that for editing in squid there is a level of control that we do not yet fully understand. For example, the physical environment could affect our results. Our in vitro assay was based on other assays that were effective for ADARs from diverse species (O'Connell et al. 1998; Ring et al. 2004; Keegan et al. 2007). Its principal components are Tris-buffered 100 mM KCl, and it was performed at 25°C. In the squid giant axon, however, the monovalent cation concentration is significantly higher, the principal anions are not chloride, there are divalent cations present, and the temperature can be significantly lower (Gilbert et al. 1990). In squid, there could also be accessory

proteins or RNAs that promote specificity, although this has not been reported for other systems. In addition, the cRNAs used for the assay contained only the channel's ORF. Perhaps sequence in the UTRs, or in sqKv1.1's sole intron, participates in secondary structures that guide editing. Finally, our cloning data show that the mRNAs that encode squid ADAR are themselves edited at multiple sites in a complex pattern. It is possible that these sites regulate the enzyme's substrate specificity. Regardless of the mechanisms for specificity, these data clearly show that sqADAR2a edits more actively than sqADAR2b.

The two squid ADARs differ by only a single dsRBD. How does this feature impart high activity? The simplest explanation is that the "extra" dsRBD is not structurally unique, but merely by having three, sqADAR2a has a higher affinity for dsRNA. It should be noted that vertebrate ADAR1 family members, which also contain three dsRBDs, have not been reported to have unusually high activity. An alternative explanation is that the "extra" dsRBD in squid is both functionally and structurally different. Previous publications show that while dsRBD1 of mammalian ADAR2 is required for dimerization and nucleolar localization, dsRBD2 is critical for activity on both specific substrates and duplex RNA (Poulsen et al. 2006; Xu et al. 2006). In these studies, deletion of dsRBD2 completely
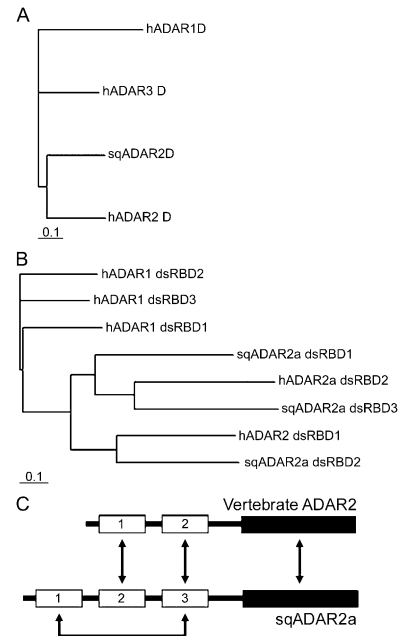

FIGURE 6. sqADAR2a is a vertebrate ADAR2 family homolog with an "extra" dsRBD. (*A*) Neighbor-joining tree comparing the deaminase domains (*D*) of sqADAR2 with those from human ADAR1, ADAR2, and ADAR3. (*B*) Neighbor-joining tree comparing the dsRBDs of sqADAR2a with those of human ADAR1 and ADAR2. Scale bars refer to 0.1 substitution per site. (*C*) Schematic representation of sqADAR2a and mammalian ADAR2. Deaminase domains and dsRBDs are represented in black and white boxes, respectively. Arrows identify the homology between the various dsRBDs.

FIGURE 7. Squid dsRBD1 has unique features. An amino acid sequence alignment of the dsRBDs from sqADAR2a and human ADAR2. Drawn *below* is a schematic of the secondary structural elements from dsRBD2 of rat ADAR2 (Stefl et al. 2006). Dark gray boxes denote identity and light gray boxes conservation.

abolished enzymatic activity, whereas for most substrates, deletion of dsRBD1 only reduced activity. Furthermore, dsRBD2 deletions could not be rescued by the insertion of dsRBD1 at its position. These experiments show that the dsRBDs in mammalian ADAR2 are not functionally equivalent, and that squid has duplicated the version most important for catalysis.

Structural studies indicate that all dsRBDs adopt a highly conserved αβββα fold (Bycroft et al. 1995; Kharrat et al. 1995; Nanduri et al. 1998). Regardless of the substrate, a dsRBD, which is stabilized by a buried hydrophobic core, makes contact with the RNA at three regions: α-helix 1, β1–β2 loop, and α-helix 2 N terminus (Ryter and Schultz 1998). The structures of the two dsRBDs from mammalian ADAR2, which were determined in complex with the GluR-B R/G stem–loop, exhibit some important differences (Stefl et al. 2006). For example, the spacing between α-helix 1 and 2 helps determine whether the dsRBD will bind to a stem–unpaired loop, as in dsRBD1, or a duplex, as in dsRBD2. Position 66 (Fig. 7), which is valine in dsRBD1 and a phenylalanine in dsRBD2, sets the spacing. sqADAR2 dsRBD1 has a phenylalanine at this position, and thus would be predicted to bind duplex regions. The two mammalian dsRBDs also differ at one of the RNA binding regions, the β1–β2 loop. dsRBD1 adopts a rigid, well-defined structure, probably due to the two prolines that flank the loop. In contrast, the β1–β2 loop of dsRBD2 is flexible and contains no prolines. Intriguingly, squid

dsRBD1 contains two prolines on the N-terminal side of the β1–β2 loop, but none on the C-terminal side. Squid dsRBD1 also differs from both of the mammalian dsRBDs at other notable positions. In α-helix 1, which contacts the RNA, it has two hydrophobic substitutions (Y7 and L12). In addition, in the α1–β1 loop there is the conspicuous insertion of a proline (P15). For all of the specific positions mentioned above, the other two dsRBDs from squid are identical to their mammalian homologs. Future experiments will determine whether the unique features of squid dsRBD1 enhance ADAR activity.

Why is recoding A-to-I RNA editing more extensive in higher invertebrates than in vertebrates? In insects, which possess a single conventional ADAR2 homolog (Palladino et al. 2000), editing site diversity is generated at the level of intronic sequences, with little or no contribution by the editing enzyme (Reenan 2005). Our data suggest that in cephalopods the situation may be different. Structural changes to ADAR itself may underlie a significant expansion of the proteome.

MATERIALS AND METHODS

Cloning of squid ADAR

Loligo opalescens specimens were collected in Monterey, California. RNA was extracted from giant fiber lobe neurons of the stellate ganglia using RNAqueous (Ambion) and used as a template for cDNA with oligo(dT) as a primer (Supersript II Invitrogen). Using degenerate PCR primers (DegF/R) (Table 3) to highly conserved motifs in the deaminase domain, a 398-nt ADAR fragment was amplified. This cDNA fragment was subsequently used as a hybridization probe to screen a squid giant fiber lobe cDNA library as previously described (Rosenthal et al. 1996). From the screen, a full-length ADAR clone was isolated and sequenced to completion (sqADAR2a). Using primers ADAR11/13, which are complementary to sequence surrounding the start and stop codons, and Pfu polymerase (Promega), full-length sqADAR2a (GenBank AN FJ478450), and sqADAR2b (GenBank AN FJ478451) were amplified from GFL cDNA and sequenced to

TABLE 3. Oligonucleotide primers used in this study

Primer	Sequence (5′ → 3′)
DegF	AAYGAYUGYCAYGCNGARATHAT
DegR	GCDATYTTRTCNGARCANGACAT
ADAR11	GTATGTCCATAAATGAGGAAAGGGG
ADAR13	AATGCGGGACTTACAGGCTA
RPAF	TGTTCAGGGTGCTGGCCGTAGT
RPAR	CCAGCTCCCTCAAAGACTTCTCC
sqNC3	AGTAATACGACTCACTATAGGGAGATCAGTATTGTGGCAGGGACGATGGG
sqNC4	AGTAATACGACTCACTATAGGGAGAATCCCCCACTTCGCTGGCAAGAC
sqKv1.2A-1	GAAATTCGGCACGAGAGCAAC
sqKv1.2A-3	CAATCATGGAGGCTTTCCCTG

Lettering follows the IUPAC convention.

completion (Fig. 1A). To clone the *sqADAR2* gene, overlapping primer pairs were designed from the cDNA sequence. Genomic DNA was isolated from gill tissue from the same *Loligo opalescens* specimen used for the cDNA and also used as a template for PCR with Pfu polymerase. Overlapping fragments were cloned and sequenced. Our attempts to amplify the intron that occurs between nucleotides 1111 and 1112 (from the cDNA's ORF) were unsuccessful. Instead, an inverse PCR strategy was used to clone the intron junctions. To identify editing sites, 50 individual cDNA clones of each variant were sequenced and compared with the gene sequence.

RNase protection assay

RNA was isolated from optic lobe and giant fiber lobe (*Loligo opalescens*) with the RNAqueous kit (Ambion). The template for the probe was amplified from sqADAR2a (positions 243–629) with the primers RPAF/R and Phusion HF DNA Polymerase (Finnzymes). After linearization with Hind III, an antisense RNA probe was transcribed from this product with T7 RNA polymerase (Promega) in the presence of 40 μCi of [α-^{32}P]UTP (3000 Ci/mmol; 1 Ci = 37 GBq) as previously described (Rosenthal and Gilly 1993). RNase protection assay was performed with RPA III Kit (Ambion) by combining 5 μg of total RNA with 10^5 cpm of probe. Samples were separated on a 5% denaturing polyacrylamide gel and exposed to a PhosphorImager for 120 h. Undigested probe samples contained 1500 cpm. Total probe length was 501 nt; protected band lengths were 387 nt and 290 nt for sqADAR2a and sqADAR2b, respectively (Fig. 3).

Purification of squid ADARs in *Pichia*

Squid ADAR splice variants were cloned into pPICZ A-FLIS6 vector and transformed into *Pichia pastoris*. Protein induction and purification have been described in detail before (Keegan et al. 2007). In this study, we used induction times of 16 h or 48 h. After disrupting the *Pichia* with a French Press, cleared lysates were first passed over a Ni^{2+}-Nitriloacetic acid column (Qiagen) and then over an α-FLAG M2 column (Sigma). Eluates were stored at −80°C in Q200 Buffer (50 mM Tris-HCl at pH 7.9, 200 mM KCl, 20% glycerol) with 1 mM DTT, 0.5 mM PMSF, 0.7 μg/mL pepstatin A, and 0.4 μg/mL leupeptin (Keegan et al. 2007). Recombinant ADARs were quantified by comparing their intensities to those of known protein standards (phosphorylase b and β-galactosidase) on an SDS-PAGE gel. Gels were stained with Krypton florescent stain (Pierce Biochemicals) and scanned on a Typhoon 9200 imager (GE Healthcare).

dsRNA editing assay

A radiolabeled dsRNA substrate was synthesized according to Ring et al. (2004) except with one difference. Instead of using the pBluescript KS-cat plasmid as a template, we used a 699-base-pair (bp) fragment from squid Na$^+$ channel GFLN1 (GenBank AN L19979, nucleotides 2110–2808). This product was amplified by PCR using the sqNC3/4 primer pair, each tagged with T7 promoters. When this fragment was used as a template for RNA production with T7 RNA polymerase, both the sense and antisense transcripts were synthesized simultaneously. For our assay, various amounts of purified squid ADARs were incubated at 30°C for 2 h with a defined amount of dsRNA substrate that contained 57 fmol ATP. After P1 nuclease treatment, samples were spotted on a Polygram TLC plates (Macherey-Nagel) and separated as previously described (Bass and Weintraub 1988). Plates were then dried, exposed overnight to a PhosphorImager screen, and scanned using a Typhoon 9200 phosphor/fluorescence imager (GE Healthcare).

Self-editing in *Pichia pastoris*

RNA was extracted from 3-mL *Pichia pastoris* cultures expressing sqADAR2a or sqADAR2b after a 16-h or a 48-h induction, with RNAqueous (Ambion). RNA was treated with DNase (Turbo DNase, Ambion) and used as a template for cDNA with oligo(dT) primer (Accuscript, Stratagene). Finally, full-length squid ADARs were amplified with Pfu Ultra II Fusion DNA Polymerase (Stratagene) and individual clones were sequenced to completion.

In vitro editing of squid K$^+$ channels cRNAs

Recombinant sqADAR2a or sqADAR2b (300 fmol) were incubated with sqKv1.1A or sqKv1.2A cRNA (20 fmol) (Molar ratio 1:15) in Q100 Buffer (50 mM Tris–HCl at pH 7.9, 100 mM KCl, 10% glycerol) with 5 mM DTT, 0.5 mM PMSF, 62.5 ng/μL tRNA, and 1 U/μL RNase OUT (Invitrogen) for 4 h at 25°C. cRNA, which contained only the channel's ORF, and was synthesized using the mScript mRNA Production System kit (Epicentre). cDNA was synthesized (Accuscript, Stratagene) after DNase treatment (Turbo DNase, Ambion). Finally, full-length K$^+$ channels were amplified with Pfu Ultra II Fusion DNA Polymerase (Stratagene) and individual clones were sequenced. To simplify data collection and analysis, we concentrated on the first 700 nt, which could be analyzed in a single sequencing run. For sqKv1.2A channel mRNAs, the complete ORF (1561 nt) was amplified with the sqKv1.2A-1/3 primer pair from giant fiber lobe cDNA. Fifty individual clones were sequenced. These data were compared with the genomic sequence, and A-to-G variations were identified.

Data analysis

For both in vivo (*Pichia*) and in vitro assays, editing percentages were estimated by sequencing 45–55 individual clones. In all cases, we considered an editing site as any position for which the gene or substrate had an adenosine and three or more clones had a guanosine. The spontaneous A-to-G conversion rate in our assays, due to a combination of reverse transcriptase, DNA polymerase (during PCR) and sequencing errors, was calculated from control reactions that contained no ADAR. It was less than one in 1748, making it highly improbable that our assignment of an editing site was due to anything but an editing event.

Sequence comparisons

Guide trees were built using the neighbor-joining method (Saitou and Nei 1987) and the AlignX program from the Vector NTI Advance Suite (Invitrogen), where distances are calculated as numbers of substitutions per site. For the dsRBD alignment, sequences were trimmed according to the complete dsRBD motif given in Stefl et al. (2006). For the deaminase domain alignment, sequences were trimmed based on the complete deaminase domain as reported in Macbeth et al. (2005).

ACKNOWLEDGMENTS

REFERENCES

Bass, B.L. and Weintraub, H. 1988. An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell* **55:** 1089–1098.

Bass, B.L., Nishikura, K., Keller, W., Seeburg, P.H., Emeson, R.B., O'Connell, M.A., Samuel, C.E., and Herbert, A. 1997. A standardized nomenclature for adenosine deaminases that act on RNA. *RNA* **3:** 947–949.

Betts, L., Xiang, S., Short, S.A., Wolfenden, R., and Carter Jr., C.W. 1994. Cytidine deaminase. The 2.3 Å crystal structure of an enzyme: Transition-state analog complex. *J. Mol. Biol.* **235:** 635–656.

Bhalla, T., Rosenthal, J.J., Holmgren, M., and Reenan, R. 2004. Control of human potassium channel inactivation by editing of a small mRNA hairpin. *Nat. Struct. Mol. Biol.* **11:** 950–956.

Burns, C.M., Chu, H., Rueter, S.M., Hutchinson, L.K., Canton, H., Sanders-Bush, E., and Emeson, R.B. 1997. Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature* **387:** 303–308.

Bycroft, M., Grunert, S., Murzin, A.G., Proctor, M., and St Johnston, D. 1995. NMR solution structure of a dsRNA binding domain from *Drosophila* staufen protein reveals homology to the N-terminal domain of ribosomal protein S5. *EMBO J.* **14:** 3563–3571.

Chang, K.Y. and Ramos, A. 2005. The double-stranded RNA-binding motif, a versatile macromolecular docking platform. *FEBS J.* **272:** 2109–2117.

Clutterbuck, D.R., Leroy, A., O'Connell, M.A., and Semple, C.A. 2005. A bioinformatic screen for novel A-I RNA editing sites reveals recoding editing in BC10. *Bioinformatics* **21:** 2590–2595.

Doyle, M. and Jantsch, M.F. 2002. New and old roles of the double-stranded RNA-binding domain. *J. Struct. Biol.* **140:** 147–153.

Gilbert, D.L., Adelman, W.J., and Arnold, J.M. 1990. *Squid as experimental animals.* New York: Plenum Press.

Gommans, W.M., Tatalias, N.E., Sie, C.P., Dupuis, D., Vendetti, N., Smith, L., Kaushal, R., and Maas, S. 2008. Screening of human SNP database identifies recoding sites of A-to-I RNA editing. *RNA* **14:** 2074–2085.

Hartner, J.C., Schmittwolf, C., Kispert, A., Muller, A.M., Higuchi, M., and Seeburg, P.H. 2004. Liver disintegration in the mouse embryo caused by deficiency in the RNA-editing enzyme ADAR1. *J. Biol. Chem.* **279:** 4894–4902.

Higuchi, M., Maas, S., Single, F.N., Hartner, J., Rozov, A., Burnashev, N., Feldmeyer, D., Sprengel, R., and Seeburg, P.H. 2000. Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature* **406:** 78–81.

Hoopengardner, B., Bhalla, T., Staber, C., and Reenan, R. 2003. Nervous system targets of RNA editing identified by comparative genomics. *Science* **301:** 832–836.

Keegan, L.P., Leroy, A., Sproul, D., and O'Connell, M.A. 2004. Adenosine deaminases acting on RNA (ADARs): RNA-editing enzymes. *Genome Biol.* **5:** 209.

Keegan, L.P., Brindle, J., Gallo, A., Leroy, A., Reenan, R.A., and O'Connell, M.A. 2005. Tuning of RNA editing by ADAR is required in *Drosophila*. *EMBO J.* **24:** 2183–2193.

Keegan, L.P., Rosenthal, J.J., Roberson, L.M., and O'Connell, M.A. 2007. Purification and assay of ADAR activity. *Methods Enzymol.* **424:** 301–317.

Kharrat, A., Macias, M.J., Gibson, T.J., Nilges, M., and Pastore, A. 1995. Structure of the dsRNA binding domain of *E. coli* RNase III. *EMBO J.* **14:** 3572–3584.

Kim, U., Wang, Y., Sanford, T., Zeng, Y., and Nishikura, K. 1994. Molecular cloning of cDNA for double-stranded RNA adenosine deaminase, a candidate enzyme for nuclear RNA editing. *Proc. Natl. Acad. Sci.* **91:** 11457–11461.

Kohler, M., Burnashev, N., Sakmann, B., and Seeburg, P.H. 1993. Determinants of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: Diversity by RNA editing. *Neuron* **10:** 491–500.

Lai, F., Drakas, R., and Nishikura, K. 1995. Mutagenic analysis of double-stranded RNA adenosine deaminase, a candidate enzyme for RNA editing of glutamate-gated ion channel transcripts. *J. Biol. Chem.* **270:** 17098–17105.

Levanon, E.Y., Hallegger, M., Kinar, Y., Shemesh, R., Djinovic-Carugo, K., Rechavi, G., Jantsch, M.F., and Eisenberg, E. 2005. Evolutionarily conserved human targets of adenosine to inosine RNA editing. *Nucleic Acids Res.* **33:** 1162–1168.

Macbeth, M.R., Schubert, H.L., Vandemark, A.P., Lingam, A.T., Hill, C.P., and Bass, B.L. 2005. Inositol hexakisphosphate is bound in the ADAR2 core and required for RNA editing. *Science* **309:** 1534–1539.

Melcher, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P.H., and Higuchi, M. 1996. A mammalian RNA editing enzyme. *Nature* **379:** 460–464.

Nanduri, S., Carpick, B.W., Yang, Y., Williams, B.R., and Qin, J. 1998. Structure of the double-stranded RNA-binding domain of the protein kinase PKR reveals the molecular basis of its dsRNA-mediated activation. *EMBO J.* **17:** 5458–5465.

O'Connell, M.A., Gerber, A., and Keegan, L.P. 1998. Purification of native and recombinant double-stranded RNA-specific adenosine deaminases. *Methods* **15:** 51–62.

Ohlson, J., Enstero, M., Sjoberg, B.M., and Ohman, M. 2005. A method to find tissue-specific novel sites of selective adenosine deamination. *Nucleic Acids Res.* **33:** e167.

Palladino, M.J., Keegan, L.P., O'Connell, M.A., and Reenan, R.A. 2000. dADAR, a *Drosophila* double-stranded RNA-specific adenosine deaminase is highly developmentally regulated and is itself a target for RNA editing. *RNA* **6:** 1004–1018.

Patton, D.E., Silva, T., and Bezanilla, F. 1997. RNA editing generates a diverse array of transcripts encoding squid Kv2 K$^+$ channels with altered functional properties. *Neuron* **19:** 711–722.

Poulsen, H., Jorgensen, R., Heding, A., Nielsen, F.C., Bonven, B., and Egebjerg, J. 2006. Dimerization of ADAR2 is mediated by the double-stranded RNA binding domain. *RNA* **12:** 1350–1360.

Reenan, R.A. 2005. Molecular determinants and guided evolution of species-specific RNA editing. *Nature* **434:** 409–413.

Ring, G.M., O'Connell, M.A., and Keegan, L.P. 2004. Purification and assay of recombinant ADAR proteins expressed in the yeast *Pichia pastoris* or in *Escherichia coli*. *Methods Mol. Biol.* **265:** 219–238.

Rosenthal, J.J. and Bezanilla, F. 2002. Extensive editing of mRNAs for the squid delayed rectifier K$^+$ channel regulates subunit tetramerization. *Neuron* **34:** 743–757.

Rosenthal, J.J. and Gilly, W.F. 1993. Amino acid sequence of a putative sodium channel expressed in the giant axon of the squid Loligo opalescens. *Proc. Natl. Acad. Sci.* **90:** 10026–10030.

Rosenthal, J.J., Vickery, R.G., and Gilly, W.F. 1996. Molecular identification of SqKv1A. A candidate for the delayed rectifier K channel in squid giant axon. *J. Gen. Physiol.* **108:** 207–219.

Rueter, S.M., Dawson, T.R., and Emeson, R.B. 1999. Regulation of alternative splicing by RNA editing. *Nature* **399:** 75–80.

Ryter, J.M. and Schultz, S.C. 1998. Molecular basis of double-stranded RNA-protein interactions: Structure of a dsRNA-binding domain complexed with dsRNA. *EMBO J.* **17:** 7505–7513.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.

Stapleton, M., Carlson, J.W., and Celniker, S.E. 2006. RNA editing in *Drosophila melanogaster*: New targets and functional consequences. *RNA* **12:** 1922–1932.

Stefl, R., Xu, M., Skrisovska, L., Emeson, R.B., and Allain, F.H. 2006. Structure and specific RNA binding of ADAR2 double-stranded RNA binding motifs. *Structure* **14:** 345–355.

Tonkin, L.A., Saccomanno, L., Morse, D.P., Brodigan, T., Krause, M., and Bass, B.L. 2002. RNA editing by ADARs is important for normal behavior in *Caenorhabditis elegans*. *EMBO J.* **21:** 6025–6035.

Wang, Q., Khillan, J., Gadue, P., and Nishikura, K. 2000. Requirement of the RNA editing deaminase ADAR1 gene for embryonic erythropoiesis. *Science* **290:** 1765–1768.

Wang, Q., Miyakoda, M., Yang, W., Khillan, J., Stachura, D.L., Weiss, M.J., and Nishikura, K. 2004. Stress-induced apoptosis associated with null mutation of ADAR1 RNA editing deaminase gene. *J. Biol. Chem.* **279:** 4952–4961.

Xia, S., Yang, J., Su, Y., Qian, J., Ma, E., and Haddad, G.G. 2005. Identification of new targets of *Drosophila* pre-mRNA adenosine deaminase. *Physiol. Genomics* **20:** 195–202.

Xu, M., Wells, K.S., and Emeson, R.B. 2006. Substrate-dependent contribution of double-stranded RNA-binding motifs to ADAR2 function. *Mol. Biol. Cell* **17:** 3211–3220.