REPORT

A-to-I RNA editing alters less-conserved residues of highly conserved coding regions: Implications for dual functions in evolution

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ABSTRACT

The molecular mechanism and physiological function of recoding by A-to-I RNA editing is well known, but its evolutionary significance remains a mystery. We analyzed the RNA editing of the Kv2 K⁺ channel from different insects spanning more than 300 million years of evolution: *Drosophila melanogaster*, *Culex pipiens* (Diptera), *Pulex irritans* (Siphonaptera), *Bombyx mori* (Lepidoptera), *Tribolium castaneum* (Coleoptera), *Apis mellifera* (Hymenoptera), *Pediculus humanus* (Phthiraptera), and *Myzus persicae* (Homoptera). RNA editing was detected across all Kv2 orthologs, representing the most highly conserved RNA editing event yet reported in invertebrates. Surprisingly, five of these editing sites were conserved in squid (Mollusca) and were possibly of independent origin, suggesting phylogenetic conservation of editing between mollusks and insects. Based on this result, we predicted and experimentally verified two novel A-to-I editing sites in squid *synaptotagmin 1* transcript. In addition, comparative analysis indicated that RNA editing usually occurred within highly conserved coding regions, but mostly altered less-conserved coding positions of these regions. Moreover, more than half of these edited amino acids are genomically encoded in the orthologs of other species; an example of a conversion model of the nonconservative edited site is addressed. Therefore, these data imply that RNA editing might play dual roles in evolution by extending protein diversity and maintaining phylogenetic conservation.

Keywords: RNA editing; phylogenetic conservation; protein diversity; insect; squid

INTRODUCTION

The most common type of RNA editing involves the conversion of individual adenosine (A) bases to inosine (I) in RNA using adenosine deaminases acting on RNA (ADAR) (Bass 2002; Maas et al. 2003). The species-specific alteration of functionally important residues in a multitude of neuronal ion channels and presynaptic proteins through A-to-I RNA editing is profoundly important for the normal function of the nervous system (Higuchi et al. 2000; Palladino et al. 2000; Wang et al. 2000; Tonkin et al. 2002). Moreover, RNA editing could influence alternative splicing

decisions (Rueter et al. 1999; Maas et al. 2001; Bratt and Ohman 2003; Flomen et al. 2004; Laurencikienė et al. 2006; Jin et al. 2007; Schoft et al. 2007), antagonize RNA interference (RNAi) (Scadden and Smith 2001; Knight and Bass 2002; Tonkin and Bass 2003), modulate miRNA processing (Yang et al. 2006; Kawahara et al. 2007a), redirect miRNA silencing targets (Kawahara et al. 2007b), and create new exons (Lev-Maor et al. 2007). In addition, the widespread editing of *Alu* elements, which compose ~10% of the human genome, plays a novel role in the primate lineage (Athanasiadis et al. 2004; Blow et al. 2004; Kim et al. 2004; Levanon et al. 2004). Overall, RNA editing plays an important role in regulating protein diversity and gene expression.

Despite progress made toward understanding the molecular mechanism and physiological function of A-to-I RNA editing, the challenge to verify its existence in evolution remains. In animals, A-to-I RNA editing mostly alters highly conserved or invariant coding positions in proteins

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.1063708>.

(Reenan 2005), thus generating protein diversity. However, there are also many examples of A-to-I RNA editing at nonconservative protein positions (Patton et al. 1997; Grauso et al. 2002; Jin et al. 2007; Ohlson et al. 2007; Tian et al. 2008). Although the phylogenetic roles of nuclear RNA editing are interesting, our understanding may not be sufficient, possibly because previous studies have used small numbers of representative animals. Therefore, we investigated the RNA editing in a broad range of animals. The squid is poised to become an important model for understanding the biological importance of RNA editing, because two examined squid K⁺ channels are heavily edited (for unknown reasons), and squid ADAR is extremely active in vitro (Patton et al. 1997; Rosenthal and Bezanilla 2002; Hoopengardner et al. 2003). We compared RNA editing between squid and insect Kv2 orthologs and found that five of these editing sites are conserved. This reveals phylogenetic conservation of editing between squid and insects. Based on these results, we predicted and experimentally verified two novel A-to-I editing sites in squid *synaptotagmin I* (*sytl*) transcript. A comparative analysis indicated that A-to-I RNA editing usually occurred in highly conserved coding regions, but mostly altered less-conserved coding positions of these regions. Moreover, more than half of these edited amino acids are genomically encoded in the orthologs of other species. Our results raise doubt about the hypothesis of Rosenthal and Bezanilla (2002) that extensive RNA editing in squid often leads to the introduction of amino acids with smaller side chains, as seen in antifreeze proteins of fish, which may increase flexibility of the molecule to compensate for cold because up to 80% of all A-to-I edits theoretically make smaller amino acids.

RESULTS

Analysis of RNA editing in the insect Kv2 K⁺ channel

Extensive A-to-I RNA editing sites were identified in the classical delayed rectifier K⁺ channel (*SqKv2*) in the squid giant axon (Patton et al. 1997), one of which occurs in both *Drosophila* Kv2 (*dShab*) and mammalian Kv1.1 channels (Bhalla et al. 2004). To determine whether Kv2 orthologs were also edited in other insect species, we analyzed the sequences of RT-PCR amplification

products to identify editing sites of three dipteran species, including *Drosophila melanogaster*, *D. virilis*, and one mosquito (*Culex pipiens*); the siphonapteran *Pulex irritans*; the lepidopteran *Bombyx mori*; the coleopteran *Tribolium castaneum*; the hymenopteran *Apis mellifera*; the phthirapteran *Pediculus humanus*; and the homopteran *Myzus persicae*. These sequence data allowed us to analyze the evolution of RNA editing over at least 300 million years.

A-to-I editing was detected across all Kv2 orthologs, but no two species possessed the same set of editing sites (Figs. 1A, 2B). One common site (site 16) was frequently edited among all nine species of seven insect orders (Fig. 1A), mostly at a frequency of more than 50%, representing the most highly conserved RNA editing site reported in invertebrates. Site 12 was conserved in *D. melanogaster*, *C. pipiens*, *B. mori*, and *A. mellifera* (Fig. 2B), but had a genomically encoded G in *P. irritans* and *P. humanus*. Site 14 was conserved across all insect species except *B. mori*

FIGURE 1. Editing in the Kv2 gene was conserved among all eight species of seven insect orders. (A) The editing sites (arrows) and their editing levels (A/G signal) among the Kv2 orthologs of *D. melanogaster* (Dme), *C. pipiens* (Cpi), *P. irritans* (Pir), *B. mori* (Bmo), *T. castaneum* (Tca), *A. mellifera* (Ame), *P. humanus* (Phu), and *M. persicae* (Mpe). RNA editing of the Kv2 genes of *D. melanogaster* have been described previously (Bhalla et al. 2004). The I/V editing site (arrows, right side of figure) is conserved among all eight species of seven insect orders. RNA editing in the synonymous site (arrows, left side of figure) is conserved among *D. melanogaster*, *C. pipiens*, *P. irritans*, *B. mori*, *T. castaneum*, and *A. mellifera*, while *P. humanus* and *M. persicae* are genomically incapable of A-to-I editing at the same location, as A is replaced by C. (B) Prediction of secondary RNA structure for the edited exon of the *D. melanogaster* Kv2 transcript using the mFold program (Zuker 2003). The edited adenosine is marked by an arrow at the I/V site. The nucleotide in *D. virilis* that differs from *D. melanogaster* is boxed.

FIGURE 2. Phylogenetic conservation of A-to-I editing in the Kv2 K⁺ channel of metazoans. (A) Conservation levels of the Kv2 K⁺ channel at the amino acid level using CLC Free Workbench 4. The high conservation level of the amino acid sequence suggests functional importance. (B) Comparison of amino acid sequences from various Kv2 orthologs encoding segment S4 through S6. Abbreviations: Hsa: *Homo sapiens* (AAA36156.1, NP_004761.2); Xla: *Xenopus laevis* (NP_001079257; NP_001079256); Dre: *Danio rerio* (XP_001332654.1; XP_001332827.1); Hro: *H. roretzi* (AB018545.1); Dme: *D. melanogaster* (NP_001036580.1); Cpi: *C. pipiens* (DS232049.1); Pir: *P. irritans* (EU616805); Bmo: *B. mori* (mb003438); Tca: *T. castaneum*; Ame: *A. mellifera* (XP_393546.3); Phu: *P. humanus* (DS235848.1); Mpe: *M. persicae* (EU616804); Lpe: *L. pealei* (CAA74748.1); Lgi: *Lottia gigantea* (Lotgi1/102260); Aca: *Aplysia californica* (AAC60504.2); Spu: *S. purpuratus* (XP_001179500); Hr: *H. robusta* (Helro1/119855, 94060, 108,240); Cca: *Capitella capitata* (Capcal/147057); Sme: *S. mediterranea* (AY066187; EG407084.1). RNA editing of the Kv2 K⁺ channel genes from squid has been described previously (Patton et al. 1997). Amino acid residues showing complete conservation are shaded in red. Residues altered by RNA A-to-I editing are shaded in green, and genomic substitutions with edited residues at these positions are shaded in blue. (C) An evolutionary tree showing the phylogenetic relationships among these metazoan species. Phylogenetic tree data were adapted and modified from Sugden et al. (2003). Residues recoded by editing are enclosed in squares. Residues encoded genomically with edited residues are circled.

and *M. persicae* (Fig. 2B), where the former was genomically incapable of A-to-I editing at the same location due to the lack of a first-position adenosine in its Ser codon (TCA) while the latter had a genomically encoded G. It is interesting to note that RNA editing occurred in the synonymous sites of *D. melanogaster*, *C. pipiens*, *P. irritans*, *B. mori*, *T. castaneum*, and *A. mellifera* (Fig. 1A), representing the most highly conserved RNA editing silent site yet reported in animals.

Because this editing site was so highly conserved, we analyzed secondary structures and an editing site complementary sequence (ECS). However, sequence alignment of the Kv2 genes failed to reveal regions of high sequence identity within upstream and downstream intronic regions surrounding the Ile/Val editing site among 12 *Drosophila* species. Nevertheless, the exonic regions flanking the Ile/Val editing site are nearly identical, and are predicted by the program mFold (Zuker 2003) to form an extended secondary structure, conserved between *D. melanogaster* and *D. virilis* (Fig. 1B; Bhalla et al. 2004). The Kv2 genes of *B. mori*, *T. castaneum*, and *A. mellifera* share limited (70%–76%) nucleotide sequence similarity with *D. melanogaster*. However, their exonic regions of the Ile/Val editing site are also predicted to form base-paired structures pairing different coding sequences than those of *Drosophila* Kv2 (data not shown). In these cases, Kv2 genes are edited at the same Ile/Val site, possibly through different exon-directed RNA secondary structures.

Comparative analysis of RNA editing between insects and squid

Insects phylogenetically belong to the class Insecta of the phylum Arthropoda, while squid is classified as a member of the Cephalopoda of the phylum Mollusca. Arthropoda and Mollusca diverged ~550 million years ago (Sugden et al. 2003). Analysis of Kv2 orthologs reveals a single copy in insects and squid, which seems to be ancestral and contains 17 editing sites occurring within a 360-nt

segment in the Kv2 K⁺ channel (Patton et al. 1997). Surprisingly, five of these sites were conserved between insects and squid, resulting in five amino acid conversions. To obtain insight into the evolution of Kv2 RNA editing, we examined RNA editing in the closely related phyla Arthropoda and Mollusca. RT-PCR analysis indicated that RNA-editing sites of insect and squid Kv2 were not conserved in *Anodonta woodiana* (class Lamellibranchia; data not shown). Expressed sequence tag and cDNA analysis of *Aplysia californica* (Gastropoda), *Lymnaea stagnalis* (Crustacea), and *Panulirus interruptus* (Crustacea) (Baro et al. 1994) failed to provide evidence for RNA editing, suggesting that RNA editing events might occur independently in insects and squid. Alternatively, multiple independent loss and gain events could possibly result in the observed RNA editing.

Prediction and verification of novel A-to-I editing sites

We demonstrated that five editing sites were conserved for the Kv2 gene between the phyla Arthropoda (insect) and Mollusca (squid), suggesting a partial role of RNA editing in maintaining phylogenetic conservation. This information can be used to predict A-to-I editing sites by identifying positions where an RNA editing event would increase evolutionary conservation. A principal candidate gene was found in the squid *synaptotagmin 1* (*sytl*) transcript. As shown in Figure 3A, sequence alignment indicated two particular amino acids (Val334 and Ala350) in the published squid *sytl* cDNA sequence (accession numbers CAA51079 and BAA09866) (Bommert et al. 1993; Mikoshiba et al. 1995), because RNA editing results in substitution of Ile to Val at the V334 ortholog site in fruit flies and mosquitoes and in substitution of Thr to Ala at the A350 ortholog site in butterflies (Reenan 2005). Therefore, we predicted that Val334 and Ala350 might have been generated by RNA editing in squid. To test this prediction experimentally, we isolated total RNA from squid and compared the sequence to the genomic sequence. An adenosine was present in the genomic sequence at the position at which a G and A mixed signal was present in the cDNA sequence (Fig. 3B). At these positions, the ACG (Thr) and AUC (Ile) codons were converted to ICG (Ala) and IUC (Val), respectively, because inosine is

read as guanosine. It is assumed that *sytl* editing events in insects represent evolutionary novelties (Reenan 2005). Verification of these two A-to-I editing sites in squid in turn indicates that RNA editing events might occur independently in insects and squid.

RNA editing usually alters less-conserved positions of highly conserved regions

Kv2 K⁺ channels are evolutionarily ancient proteins conserved throughout the animal kingdom. We deduced the amino acid (AA) sequence of each Kv2 protein in Platyhelminthes, Annelida, Mollusca, Arthropoda, Echinodermata, and Chordata. Comparative analysis of the AA sequences revealed that there were several evolutionarily conserved sequences with the highest conservation in the S1-6 domain (Fig. 2A). The similarity of the AA sequences of any two species was >70%, and there were 73 absolutely conserved AA (Fig. 2B, shaded in red) within the S4-6 region (122 AA). The conservation in this region was

FIGURE 3. Two novel A-to-I editing sites were predicted and experimentally verified in squid *sytl*. (A) Comparison of amino acid sequences from various *sytl* orthologs. Abbreviations: Hsa: *H. sapiens* (AAA60609; BAC04354); Xla: *X. laevis* (CX838804, CX794956); Dre: *D. rerio* (NP_001018382, XM_683000.2; XM_691185.2); Lpe: *L. pealei* (CAA51079.1); Dme: *D. melanogaster* (AAF51205); Aga: *A. gambiae* (XP_317535); Bmo: *B. mori* (Scaffold005935); Tca: *T. castaneum* (XP_969399); Mse: *Manduca sexta* (AAK01129); Ame: *A. mellifera* (XP_392154); Aca: *A. californica* (AAQ91785; P41823); Lst: *L. stagnalis* (AAO83847); Dom: *Discopyge ommata* (AAA49228.1); Pdu: *Platynereis dumerilii* (ABR68850.1); Dja: *Dugesia japonica* (BAA85622); Spu: *S. purpuratus* (AAB67801.3); Hro: *H. robusta* (BAB18864); Cel: *Caenorhabditis elegans* (NP_001022129). RNA editing of the *sytl* in insects has been described previously (Reenan 2005). Amino acid residues showing complete conservation are shaded in red. Residues recoded by RNA editing are shaded in green, and genomic substitutions with edited residues at these positions are shaded in dark blue. Two candidate residues (shaded in dark blue) altered by RNA editing are predicted in squid. (B) Two editing sites in squid (*Loligo chinensis*) *sytl* are experimentally verified and compared to fruit fly (*D. melanogaster*) and silkworm (*B. mori*). RNA editing results in substitution of Ile to Val at the V334 ortholog site in the fruit fly, and in substitution of Thr to Ala at A350 ortholog site in the silkworm. Reverse transcriptase reactions were performed with oligo(dT)₁₅ on total RNA from squid. The RT-PCR and PCR products were purified for direct sequencing. The editing sites (arrow) showed an A/G mixed signal, while the DNA sequence exhibited only adenosine signals.

markedly higher than in the other regions. Considering the fact that most editing sites were in this conserved region, it is tempting to think that A-to-I editing usually occurred in highly conserved coding regions.

However, RNA editing resulted in 16 AA changes in insects and squid (Patton et al. 1997), of which only one is completely conserved in 19 species, from low-level invertebrates (*Schmidtea mediterranea*) to vertebrates (human) (Fig. 2B). In this case, A-to-I editing at these sites mostly occurred at relatively less-conserved positions. An extensive analysis of known fruit fly editing genes also showed that editing occurred extensively at relatively less-conserved positions of the highly conserved region (Supplemental Fig. S1). The situation is similar in vertebrates, as the most intensively studied example of GluR-B RNA editing, the Q/R site, is located at a relatively less-conserved position of the highly conserved transmembrane domain 2 (TM2) (Kung et al. 1996). The I/M editing site of the GABA_A receptor subunit $\alpha 3$ is another example, as editing occurred at relatively less-conserved positions of the highly conserved TM3 (Ohlson et al. 2007). These data suggest that edited sites accumulate mutations much more rapidly than neighboring unedited sites. This idea is strengthened by recent analyses of RNA editing of nAChR $\alpha 6$, which suggested that edited sites accumulate mutations nearly twice as rapidly as unedited sites (Tian et al. 2008). These results contrast with previous reports in *Drosophila*, in which A-to-I RNA editing mostly altered highly conserved or invariant coding positions in proteins (Reenan 2005). However, these previous studies had limited sample sizes and compared closely related species. Our results indicate that, overall, RNA editing usually alters less-conserved positions of highly conserved regions, although some absolutely conserved residues are recoded by editing (Rosenthal and Bezanilla 2002; Reenan 2005; Jepson and Reenan 2007).

Edited residues are frequently DNA encoded in other species

We analyzed the amino acids at nonconservative editing positions. Interestingly, more than half of these edited amino acids were genomically encoded in the orthologs of other species (Fig. 2B,C). For example, editing results in the conversion of Ile to Val at site 2 in squid, but is genomically encoded in *D. melanogaster*, *C. pipiens*, *B. mori*, and *T. castaneum* (Fig. 2B,C). RNA editing at site 3 also recodes Ile to Val in squid, but is genomically encoded in *Strongylocentrotus purpuratus* and *Helobdella robusta* (Fig. 2B,C). Furthermore, editing at site 11 recodes Thr to Ala in the fruit fly and mosquito, but is genomically encoded in *S. mediterranea* (Fig. 2B,C). In addition, conversion of Ile to Val by RNA editing occurs at site 4 in squid and site 5 in fruit flies, but these are genomically encoded in *S. mediterranea* (Fig. 2B,C). A further A-to-I editing event results in substitution of Ile to Val at site 16 in squid and insects,

but this is genomically encoded in *S. mediterranea* and *H. robusta* (Fig. 2B,C). These data suggest that RNA editing in squid and insects might help maintain phylogenetic conservation.

The possibility of phylogenetic conservation by RNA editing is strengthened by an analysis of squid Kv1 K⁺ channels, which are heavily edited (Rosenthal and Bezanilla 2002). Analysis of Kv1 orthologs from invertebrate and vertebrate species indicates that more than half of the edited amino acids in squid are genomically encoded in other species (Fig. 4A,B). A-to-I RNA editing results in extensive substitution of Ile to Val in squid, but this is genomically encoded in most vertebrate and invertebrate species (Fig. 4B). Analysis of Kv1 orthologs indicates that this subfamily mostly contains just a single member in most invertebrates, whereas an expanded Kv1 subfamily exists in vertebrates such as human and mouse (seven members), chicken (at least six members), and zebrafish (at least six members) (Fig. 4A). Further analysis of Kv1 orthologs reveals that Ile and Val are located at these ortholog sites in vertebrates. Considering their similar residues at these sites, it is tempting to speculate that the function of these residues is expanded by gene duplication and divergence in vertebrates; while squid and fruit flies employ RNA editing to expand their function, although their function might be affected by other residues (Jepson and Reenan 2007).

An evolutionary example of a nonconservative edited site

We found that one conserved editing site (Fig. 2B, site 14) is located in a nonconserved residue genomically encoded by Gly, Ser, Thr, and Ala in metazoans. Notably, according to amino acid structure and characteristics, Gly and Ser are similar to Thr and Ala, respectively (Fig. 5). Phylogenetic analysis in insects indicates that Ser represents the ancestral state at this site, at least before the radiation of Diptera and Lepidoptera (spanning ~250 million years). At the divergence of these two groups, Ser was converted to Thr in Diptera but remained Ser in Lepidoptera. Analysis of the orthologs of 12 *Drosophila* species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis*, and *D. grimshawi*) and three mosquito species (*Anopheles gambiae*, *C. pipiens*, *Aedes aegypti*) as well as the siphonapteran *P. irritans* showed that they possess a Thr at this site. Interestingly, this Kv2.1 homolog site has a genomically encoded Gly in all known vertebrates (Fig. 2B). Similar to squid (Patton et al. 1997), A-to-I RNA editing at this site results in substitution of Ser to Gly in *T. castaneum*, *A. mellifera*, *P. humanus*, which is genomically encoded in *M. persicae* and vertebrates (Fig. 2B). Conversely, editing at this site in fruit flies and mosquitoes recodes Thr to Ala, which is genomically encoded in *Halocynthia roretzi* and *H. robusta* (Fig. 2B,C). In summary,

FIGURE 4. Phylogenetic conservation of A-to-I editing in the Kv1 K⁺ channel of metazoans. (A) Alignment of the amino acids of the Kv1 K⁺ channel genes. Abbreviations: Dme: *D. melanogaster* (NP_728123); Lop: *L. opalescens* (AAB02884); Spu: *S. purpuratus* (XP_001178924); Aga: *A. gambiae* (XP_310869); Tca: *T. castaneum* (XP_968511); Ame: *A. mellifera* (XP_391895); Aca: *A. californica* (AAA27756); Hsa: *H. sapiens* (NP_000208; BAF82750; NP_002223; NP_002224; NP_002225; NP_114092; NP_005540); Gga: *Gallus gallus* (XP_425660; NP_989794; NP_001025549; XP_417226; NP_989793); Dre: *D. rerio* (XP_687507; XP_001334455; XP_699869; NP_001104640; XP_695984; XP_687427). RNA editing of the Kv1 K⁺ channel genes of *D. melanogaster* and squid has been described previously (Rosenthal and Bezanilla 2002; Hoopengardner et al. 2003). The residues altered by RNA editing sites are shaded in black, and genomic substitutions with edited residues at these positions are shaded in gray. (B) An evolutionary tree showing the phylogenetic relationships among species. Residues recoded by RNA editing are enclosed in squares.

there are four alternative conversions for Ser (Fig. 5). In vertebrates, Ser was converted to Gly at the DNA level, while RNA editing recoded Ser into Gly in invertebrates such as squid, *T. castaneum*, *A. mellifera*, and *P. humanus*. In the dipterans *D. melanogaster* and *C. pipiens* and the siphonapteran *P. irritans*, Ser evolved into Thr at the DNA level. Thr was then converted to Ala by subsequent RNA editing while Ser was converted to Ala at the DNA level in sea squirts (*H. roretzi*). Interestingly, analysis of Kv2 orthologs reveals Ser and Ala at these equivalent positions in the *H. robusta* Kv2 family (Fig. 2B) and thus may resemble Ser and Gly generated by RNA editing in some insect species. This substitution by RNA editing provides an evolutionary model for extending protein diversity and maintaining phylogenetic conservation.

This conversion model of nonconservative edited sites also appears in other genes. For example, four residues (Gly, Ser, Thr, and Ala) are also located at the equivalent position of edited Thr350 in squid *sytl* (Nakhost et al. 2004). Based on the structural similarity of Ser and Thr and

Ala and Gly, switching these amino acids should not result in a major structural change while substitution between Ser/Thr and Ala/Gly could potentially result in a major functional change. For example, RNA editing generates a Ser/Gly substitution close to the fruit fly ADAR active site, which could result in less active ADAR than the genome-encoded, unedited isoform (Keegan et al. 2005). A Thr/Gly mutation at the equivalent position of *sytl* at the edited site shows greater resistance to proteolysis in the absence of calcium (Nakhost et al. 2004). Therefore, species-specific expansion at this site in DNA and RNA seems to have adaptive functional significance.

DISCUSSION

The most highly conserved RNA editing event yet reported in invertebrates

In invertebrates, most known RNA editing events are reported in *Drosophila*, and these editing sites are mostly

FIGURE 5. Conversion model of the nonconservative edited site (Fig. 2B, site 14) in Kv2 at the DNA and RNA level. Ser might represent the ancestral state at this position, and there are four alternative conversions for Ser. In most vertebrates, Ser was converted to Gly at the DNA level, while editing recoded Ser into Gly by RNA in invertebrates such as squid, *T. castaneum*, *A. mellifera*, and *P. humanus*. In the dipterans *D. melanogaster* and *C. pipiens* as well as the siphonapteran *P. irritans*, Ser was converted to Thr at the DNA level, and then to Ala by RNA editing, while Ser evolved into Ala at the DNA level in sea squirt (*H. roretzi*) and *H. robusta*. Notably, Gly and Ser are structurally similar to Thr and Ala, respectively.

confined to Diptera. In addition to Kv2, there are only two examples of RNA editing being conserved beyond Diptera. One example is *sytl*, for which fruit flies, dipteran mosquitoes, and butterflies (Lepidoptera) share one editing site, whereas honeybees and beetles do not edit *sytl*. Another example is nAChR alpha6, for which two editing sites are conserved in four insect orders, represented by *D. melanogaster*, *B. mori*, *T. castaneum*, and *A. mellifera* (Grauso et al. 2002; Jones et al. 2006; Jin et al. 2007), while mosquitoes do not edit nAChR alpha6 (Jones et al. 2005). In the present study, site 16 in Kv2 was conserved among seven orders, represented by *D. melanogaster*, *P. irritans*, *B. mori*, *T. castaneum*, *A. mellifera*, *P. humanus*, and *M. persicae* (Figs. 1A, 2B). RNA editing also occurred at this equivalent position in squid (Patton et al. 1997). Therefore, this represents the most highly conserved RNA editing event yet reported in invertebrates.

Conversion of Ile to Val at this site by RNA editing alters the rates of channel closure and inactivation in squid (Patton et al. 1997). In addition, the editing event (Ile/Val) profoundly affects channel inactivation conferred by accessory β -subunits at the equivalent position of human Kv1.1, while *D. melanogaster* Kv1 channels exhibit a similar effect through Ile/Val mutation (Bhalla et al. 2004). Interestingly, analysis of Kv2 orthologs revealed Ile as well as Val residues at these equivalent positions in the *H. robusta* Kv2 family (Fig. 2B,C), indicating that Ile-to-Val conversion occurs via gene duplication and divergence, thus mimicking this editing event. Therefore, it seems likely that Ile-to-Val conversion at this site has adaptive significance.

Species-specific synonymous mutation potentially alters amino acids by RNA editing

Site 14 in Kv2 was conserved in *D. melanogaster*, *C. pipiens*, *P. irritans*, *T. castaneum*, *A. mellifera*, and *P. humanus* but not *B. mori* (Fig. 2B,C). A similar situation occurred for nAChR alpha6, for which two editing sites were conserved in four orders, represented by *D. melanogaster*, *B. mori*, *T. castaneum*, and *A. mellifera* (Grauso et al. 2002; Jones et al. 2006; Jin et al. 2007), while editing was undetectable in dipteran mosquitoes. However, silkworm Kv2 lost this editing site while the two neighboring sites were edited. Thus, this codon might be located within a secondary structure, which would be necessary for the editing of the two neighboring sites. Because an ECS might form entirely from a conserved exonic sequence, it is very difficult to change ECS to escape editing. Therefore, codon synonymous mutation, which results in a lack of edited adenosine, might be a mechanism for escaping editing. This AGY-to-TCN synonymous substitution is also detected in other Lepidopteran insects, such as *Epiphyas postvittana* (EV813217) and *Antheraea pernyi* (data not shown). Editing in these species might be unnecessary and even deleterious, and thus AGY-to-TCN substitution can genomically escape this RNA editing. Further experiments are necessary to verify whether synonymous mutations from TCN to AGY can restore this RNA editing. Our observations imply that species-specific codon synonymous mutations might help regulate changes to amino acids via RNA editing.

Implications for dual function of RNA editing in evolution

Comparative analysis indicated that A-to-I editing usually occurred in highly conserved coding regions, but usually recoded less-conserved coding positions of these regions. Furthermore, more than half of these edited amino acids are genomically encoded in the orthologs of other species. These results suggest that RNA editing plays dual roles in evolution: extending protein diversity and maintaining phylogenetic conservation. Our analyses indicate that edited sites accumulate mutations much more rapidly than unedited sites, and thus evolution of sites undergoing mRNA editing is accelerated to enhance protein diversity. In addition, the RNA editing system is driven by genetic variation to maintain phylogenetic conservation, similar to plant mitochondrial RNA editing. In plants, RNA editing results in increased similarity with respect to homologous protein sequences among different organisms (Covello and Gray 1989; Gualberto et al. 1989; Tillich et al. 2005; Mulligan et al. 2007), suggesting that RNA editing helps repair otherwise deleterious genomic mutations (Gray and Covello 1993). Unlike most cases of plant RNA editing, however, animal genes are seldom fully edited. Much

TABLE 1. Primers used for the RT-PCR and PCR analysis

Species	Primer name	Primer sequence (5'–3')
<i>D. melanogaster</i>	Dmekv2-5-1	AGAAGTATCTGTGGGAGCTCCTCGA
	Dmekv2-3-1	GATCCATGGACTTGGCGAAGGCATC
<i>D. virilis</i>	Dvikv2-5-1	GGAGTTCGGCGAAGGTAATGCGC
	Dvikv2-3-1	TGATATGATGGAAGGAGACAATGCT
<i>C. pipiens</i>	Cpkv2-5-1	AGCGATGACCTCGAGTACTGGGGCGT
	Cpkv2-5-2	CAGCGGGAGGAGGAAGAGTT
	Cpkv2-3-1	TTGATATGGTGAAGCTGACGATGCT
	Cpkv2-3-2	GCCTTCTCGCGGCGCATCTG
<i>B. mori</i>	BmKv2-5-1	TGGGACTTATTGGAGAAGCCGA
	BmKv2-3-1	GTCCATGCTCTTGGCGAAAGCGT
	BmKv2-5-2	GGGCACATGTGCTCAATATC
	BmKv2-3-2	CTCGTAGGTTTATGTGGTGG
<i>T. castaneum</i>	TcaKv2-5-1	AGTGGCTCTGGGACATGCTCGAG
	TcaKv2-3-1	CAATGACGTCGATCAGGTCCATGCT
	TcaKv2-5-2	AACCGACCACCTCGATCGCG
	TcaKv2-3-2	CATCAACTTGAGGGACGCCTT
<i>A. mellifera</i>	AmeKv2-5-1	TCAGAAATGGCTATGGGATTGCT
	AmeKv2-3-1	CGACGATGACATCGATGAGATCCAT
	AmeKv2-5-2	GAGAAACCAACTACGTCCATC
	AmeKv2-3-2	CGAAAGCGTCCCTCAGATTG
<i>P. humanus</i>	PhKv2-5-1	TCTCGAATATTGGGGAGTCG
	PhKv2-5-2	CGTGTGTGCAGATAAGTACC
	PhKv2-3-1	TGTGTCCACGATGACGTCAAT
	PhKv2-3-2	TGCTCTTGGCGAAAGCGTCC
<i>Loligo chinensis</i>	LpeSyn-5-1	TATCGTTGATGCTTAACGG
	LpeSyn-3-1	CTGTATTGTTCGAACGGGAC
Degenerate primer	DeKv2-5-1	ATGGTNGARGCNGTNTGYATCACNTGGTT
	DeKv2-5-2	AGTGCCTCRCCSGAYAARTGGAA
	DeKv2-3-1	GTCCATRCTYTTSCCGAANGCRTC
	DeKv2-3-2	CATCTGRTTYTTTARAAYTCRGC

N: A/T/C/G R: A/G Y: C/T S: C/G.

evidence indicates that unedited RNA is biologically important (Vissel et al. 2001). It is possible that partial RNA editing may be sufficient to maintain phylogenetic conservation. If so, this would support our implications for the dual function of RNA editing in evolution.

Our results raise doubt about the hypothesis of Rosenthal and Bezanilla (2002) that extensive RNA editing in squid often leads to the introduction of amino acids with smaller side chains. However, this is not surprising, because up to 80% of all A-to-I edits theoretically make smaller amino acids. Three bases in a DNA or RNA sequence specify a single amino acid according to a standard genetic codon. There are 64 triplet codons, 37 of which include A. In theory, these codons may produce 61 new codons by A-to-I editing, 35 of which result in amino acid substitution. Comparative analysis indicates that 80% of the edits (28/35) make smaller amino acids. Of the remaining 20%, three substitutions [ATA(Ile) → ATI(Met); CAT/C(H)CIT/C(R)] change the size very little. The four examples of size increase are CAA/G(Q) to CIA/G(R) and AAA/G(K) to

AIA/G(R) conversions. A model of the evolutionary mechanism of nuclear A-to-I RNA editing as an evolutionary intermediate of genetic variation was proposed (Tian et al. 2008). According to this model, Val-to-Ile mutation in sites 4, 9, and 12 of Kv1 might occur in squid (Fig. 4B) and subsequent A-to-I RNA editing at the homologous site may counteract this conversion, thus maintaining similarity at the protein level. Ile-to-Val mutation in sites 7, 8, and 10 of Kv1 might occur in vertebrates, mimicking RNA editing at these sites in squid (Fig. 4B). Although single editing events may often produce a protein that is not simply intermediate in character between fully edited and unedited proteins (Jepson and Reenan 2007), there are some examples of DNA mutation antagonizing RNA editing (Higuchi et al. 2000; Bhalla et al. 2004). Either way, RNA editing is more tolerated at these positions, as these edited residues are most commonly found at the ortholog positions of other species. Therefore, extensive RNA editing in squid might help maintain phylogenetic conservation rather than produce smaller amino acids.

MATERIALS AND METHODS

Materials

Human head lice (*P. humanus*) and fleas (*P. irritans*) were collected from the countryside, Zhejiang Province, China. Green peach aphid (*M. persicae*) was donated from the Mingguang Feng laboratory, Zhejiang University. Other insect materials were obtained as previously reported (Jin et al. 2007; Tian et al. 2008). Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Genomic DNA was isolated using the Universal Genomic DNA Extraction Kit (TaKaRa). Plasmid DNA was purified using the Qiagen plasmid isolation kit.

Identification and cloning of the Kv2 genes of insects

The sequences of the Kv2 K⁺ channel gene of *D. melanogaster* were described previously (Butler et al. 1989). To identify putative genes encoding the Kv2 K⁺ channel, we screened the genomes of *C. pipiens* (mosquito; Diptera), *B. mori* (silkworm; Lepidoptera), *T. castaneum* (red flour beetle; Coleoptera), *A. mellifera* (honeybee; Hymenoptera), and *P. humanus* with *D. melanogaster* Kv2 K⁺ channel using the BLAST algorithm. Candidate Kv2 K⁺ channels were identified based on their considerable sequence homology with *D. melanogaster* Kv2 K⁺ channels. For *P. irritans*, *M. persicae*,

and *A. woodiana*, degenerate primers were necessary to amplify products (Table 1). Amplification products were cloned into the pGEM-T Easy vector for sequencing. The nucleotide and amino acid sequences from each species were aligned using Clustal W.

Analysis of RNA editing

Total RNA was isolated from adult insects using the RNeasy Mini Kit (Qiagen). RNA preparations were digested with RNase-free DNase I (TaKaRa) to ensure the absence of genomic DNA contamination. First-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's protocol. RT-PCR using the proof-reading pfu DNA polymerase (TaKaRa) was performed using the primer pairs for the Kv2 gene in different species. The primers used for amplification of RT-PCR and PCR products are shown in Table 1. RT-PCR amplicons were directly sequenced after gel purification. The genomic PCR amplification products were also directly sequenced to demonstrate that genomic products give a pure A signal at editing sites, ruling out polymorphism. In addition, the RT-PCR products were cloned into the pGEM-T Easy vector (Promega), and approximately 20 cDNA clones were sequenced for every sample to determine relative A-to-G abundance.

SUPPLEMENTAL DATA

Supplemental material can be found at <http://www.rnajournal.org>.

ACKNOWLEDGMENTS

We acknowledge Julia Hosp for help in the writing of the manuscript. This work was partly supported by research grants from the National Natural Science Foundation of China (90508007, 30770469), and 863 Program (2006AA10A119) and the Program for New Century Excellent Talents in University (NCET-04-0531).

Received March 6, 2008; accepted April 16, 2008.

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