RNAi is antagonized by A→I hyper-editing

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RNA interference (RNAi) and adenosine to inosine conversion are both mechanisms that respond to double-stranded RNA (dsRNA) and have been suggested to have antiviral roles. RNAi involves processing of dsRNA to short interfering RNAs (siRNAs), which subsequently mediate degradation of the cognate mRNAs. Deamination of adenosines changes the coding capacity of the RNA, as inosine is decoded as guanosine, and alters the structure because A–U base pairs are replaced by I•U wobble pairs. Here we show that RNAi is inhibited if the triggering dsRNA is first deaminated by ADAR2. Moreover, we show that production of siRNAs is progressively inhibited with increasing deamination and that this is sufficient to explain the inhibition of RNAi upon hyper-editing of dsRNAs.

INTRODUCTION

Extended double-stranded RNA (dsRNA) duplexes are unusual in eukaryotic cells and are often indicative of viral infection or the presence of other mobile genetic elements. A number of mechanisms exist to respond to dsRNA. For example, the PKR and RNase L/oligo 2-5 A synthase systems act to globally inhibit gene expression in vertebrate cells (Silverman *et al.*, 1997; Samuel, 1998). In contrast, RNA interference (RNAi) and adenosine deamination by ADARs (adenosine deaminases that act on dsRNA) directly target the dsRNA with covalent processing reactions and coexist throughout the metazoa (Bass, 2000; Hough and Bass, 2000; Carthew, 2001; Sharp, 2001).

RNAi is the post-transcriptional gene silencing (PTGS) mechanism whereby dsRNA directs the specific degradation of cognate mRNA (see Bass, 2000; Carthew, 2001; Sharp, 2001 for reviews). Following the discovery of RNAi in *Caenorhabditis elegans* (Fire *et al.*, 1998), it was subsequently observed in a variety of eukaryotic organisms. Moreover, parallels have been drawn between RNAi and the PTGS mechanisms of co-suppression in plants and quelling in fungi. PTGS in plants has been associated with antiviral defence (Hamilton and Baulcombe, 1999). It is likely that RNAi exists to counteract invasion of cells

by mobile genetic elements such as viruses, transposable elements or transgenes. Degradation of the target mRNA is restricted to the region that corresponds to the dsRNA (Zamore *et al.*, 2000). Initially, the dsRNA is processed to generate short RNA fragments of 21–23 nt, referred to as small interfering RNAs (siRNAs) (Zamore *et al.*, 2000; Elbashir *et al.*, 2001). The highly conserved class of RNase III homologues, represented by the *Drosophila* enzyme *Dicer*, have been implicated in processing of dsRNA to siRNAs (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Knight and Bass, 2001). Subsequently, the siRNAs are thought to act as guide sequences within a multicomponent nuclease for targeted degradation of the mRNA (Hammond *et al.*, 2001).

dsRNAs are also targets for hyper-editing by ADARs (Emeson and Singh, 2000; Hough and Bass, 2000). ADARs catalyse the hydrolytic deamination of adenosine (A) to inosine (I). This has two important consequences. First, the coding capacity of the RNA is changed, since I is recognized as G during translation. Secondly, the structure of the dsRNA is altered by the replacement of Watson–Crick A–U base pairs by I•U wobble pairs, which are isomorphic with G•U base pairs (Masquida and Westhof, 2000). The change in coding capacity is important during specific editing of mRNAs, in which a limited number of A→I changes, guided by short intramolecular base-paired segments, alter specific amino acids within the encoded protein. In contrast, hyper-editing of long dsRNA results in up to 50% of adenosine residues being replaced by inosine, resulting in multiple mis-sense alterations. Moreover, the specific structures induced by the wobble I•U base pairs or the presence of inosine *per se* may also tag the dsRNA for specific degradation (Scadden and Smith, 2001) or nuclear retention (Zhang and Carmichael, 2001).

Although both RNAi and ADARs are thought to have antiviral roles, it is likely that the two processes are mutually antagonistic. Conversion of dsRNA to siRNA would antagonize the activity of ADARs, because siRNA duplexes are expected to be too short for adenosine deamination (Hough and Bass, 2000). Likewise, hyper-editing could antagonize RNAi at two levels (Bass, 2000). First, hyper-editing by ADARs is likely to alter the structure of the

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dsRNA sufficiently that it is a poor substrate for siRNA production. Secondly, the mRNA targeting step of RNAi might be inhibited by the substitution of I•U for A–U base pairs between the siRNAs and the target mRNA. Finally, I•U base pairs within the siRNA duplexes themselves might be inhibitory for RNAi. We have now tested the proposed antagonism between ADARs and RNAi. We show that hyper-editing by ADAR2 antagonizes RNAi *in vitro* and that the inhibition of RNAi is accompanied by a significant decrease in the production of siRNAs from deaminated dsRNA. This suggests that resistance to RNAi in some tissues (e.g. neuronal cells in *C. elegans*) could be explained in part by high ADAR activity.

RESULTS AND DISCUSSION

RNAi is antagonized by A→I editing

We investigated RNAi *in vitro* using the *Drosophila* extract described previously (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). The target mRNA comprised ~850 nt of the bacterial chloramphenicol acetyl transferase (CAT) gene at a concentration of 50 pM. The homologous dsRNA used to trigger RNAi was 595 bp in length beginning at the 5' end of the target. A deaminated-dsRNA (d-dsRNA) trigger was prepared by hyper-editing *in vitro* with recombinant ADAR2 to give ~50% A→I conversion. Finally, a control trigger single-stranded RNA (ssRNA) corresponded to the sense strand of the CAT target. Typically, the trigger RNAs were uniformly labelled at a very low specific activity and were added to 10 nM. While the CAT target RNA was degraded at a similar rate to a control ssRNA (ΔKP) in the presence of single-stranded CAT trigger RNA (Figure 1A, lanes 9–12), in the presence of dsRNA trigger the CAT RNA, but not the control, was degraded at an enhanced rate [lanes 1–4, and illustrated graphically in Figure 1B (squares)]. In contrast, the d-dsRNA trigger (~50% deamination) was completely inactive in RNAi [Figure 1A, lanes 5–8, Figure 1B (circles)]. Even when the time-course was extended to 180 min, no significant RNAi activity was observed with d-dsRNA (data not shown). To ensure the specificity of the *in vitro* RNAi, the assay was repeated using three different control RNAs: VLE (445 nt), ΔKP (300 nt) and PV1 (230 nt) (Figure 1C and D). The degradation of the target RNA (with either dsRNA or d-dsRNA) was quantitated relative to each of the control RNAs and normalized to the ratio of target/control degradation observed in the presence of 10 nM ssRNA (Figure 1D). These data confirm that, while dsRNA is potent in inducing RNAi, d-dsRNA is inactive.

In the experiments shown in Figure 1, the d-dsRNA was maximally edited, with ~50% of A residues converted to I. RNAs edited more moderately would have a less disrupted structure and may be partially active in RNAi. We therefore tested the ability of d-dsRNAs edited to various levels to activate RNAi and to generate siRNAs (Figure 2). The amount of A→I conversion was quantitated by phosphorimaging following digestion with RNase P1 and TLC (data not shown). The d-dsRNAs subsequently used in the RNAi assays contained 5, 12, 19, 25 and 43% A→I conversion. The d-dsRNAs were analysed by native gel electrophoresis to verify that all of the input RNA was deaminated to a similar extent, as indicated by the relatively

Fig. 1. RNAi in *Drosophila* extract. (A) Unlabelled RNAs (dsRNA, d-dsRNA and ssRNA) were assayed for their ability to activate RNAi. These RNAs (10 nM) were preincubated in *Drosophila* extract for 15 min at 25°C before adding 50 pM of each of the target and control RNAs (CAT and ΔKP, respectively). The assay was continued for a further 15, 30 or 45 min and the products analysed on a 5% polyacrylamide gel. While dsRNA was able to activate RNAi (lanes 1–3), neither d-dsRNA or ssRNA were active. (B) The data from (A) is expressed as a ratio of CAT/ΔKP [+dsRNA (squares) or +d-dsRNA (circles)] relative to the ratio of CAT/ΔKP +ssRNA. The graphical representation of these data reinforces the observation that d-dsRNA is unable to activate RNAi. (C) dsRNA, d-dsRNA and ssRNA (10 nM) were again tested for their ability to activate RNAi. Three different control RNAs (VLE, ΔKP and PV) and the target RNA (CAT) were used (50 pM each RNA). Time points were taken at 0, 30 and 60 minutes. Again, only dsRNA was able to activate RNAi (lanes 1–3). Note that the ~600 nt band in this panel represents the trigger RNA, which was labelled to higher specific activity than in the other experiments. (D) The data from (C) were expressed as a ratio of CAT/control [(+dsRNA or d-dsRNA) for each control RNA] relative to the ratio of CAT/control (+ssRNA) for each control RNA: CAT/VLE (triangles), CAT/ΔKP (squares), CAT/PV (circles). Data-points for dsRNA are joined by solid lines, and for d-dsRNA by dashed lines. Approximately the same RNAi activity was observed regardless of the control RNA used to calculate the CAT/control ratio and the d-dsRNA was again ineffective at activating RNAi.

tight bands with lower mobility than unmodified dsRNA (Figure 2A). An RNAi assay was carried out for 90 min where 10 nM CAT dsRNA, ssRNA or each d-dsRNA was used to activate RNAi against the CAT target in the presence of an unrelated control RNA (ΔKP) (Figure 2B). The data were quantitated and are represented graphically in Figure 2C, as the ratio of remaining CAT/ΔKP RNAs normalized to the CAT/ΔKP ratio in the presence of ssRNA. Again dsRNA but not ssRNA trigger caused a decrease in the amount of CAT target RNA (compare lanes 1 and 7). As the amount of editing of the d-dsRNA increased, a corresponding decrease in its effectiveness to induce RNAi was seen (lanes 2–6). Thus, increasing levels of deamination progressively impair the ability of d-dsRNA to induce RNAi.

Fig. 2. A→I editing progressively inhibits RNAi and production of siRNAs. (A) CAT dsRNA was edited to varying degrees (5, 12, 19, 25 and 43% A→I) using ADAR2 and analysed by native gel electrophoresis. As the level of editing increased there was a corresponding reduction in their mobility on a native gel (for example, compare lanes 1 and 6). Each deaminated RNA was relatively homogeneous, as indicated by the relatively tight bands with lower mobility than unmodified dsRNA. (B) An RNAi assay was performed for 90 min where each CAT d-dsRNA was tested for its ability to activate RNAi (lanes 2–6) alongside dsRNA (lane 1) and ssRNA (lane 7). In this assay the control RNA used was ΔKP. As the level of editing increased in the d-dsRNAs, there was a clear reduction in the amount of RNAi (compare lanes 1 and 6). (C) The data from (B) are represented as the CAT/ΔKP ratio relative to the CAT/ΔKP ratio in the presence of ssRNA. In order to accurately quantitate the amount of target RNA remaining in the presence of highly edited d-dsRNA (lanes 4–6), it was necessary to determine the sum of the two upper bands that correspond to CAT RNA (marked with an asterisk in lane 6). The anomalous mobility of a small proportion of the CAT target RNA is the result of partial hybridization of the CAT target RNA with the 200-fold excess of unlabelled CAT d-dsRNA. An inverse correlation exists between the level of editing in d-dsRNA and its ability to induce RNAi. (D) The production of siRNAs from CAT dsRNA and d-dsRNAs was analysed using RNAs labelled with [α - 32 P]ATP. This assay was performed alongside that shown in (B) using identical conditions. As the level of editing increased, the production of siRNAs showed a corresponding decrease (compare lanes 1 and 6). The percentage of the input RNA that was converted to siRNAs is shown below each lane.

Editing of RNA reduces the production of siRNAs

To test whether hyper-editing directly inhibits the production of siRNAs, we labelled trigger RNAs to high specific activity with

[α - 32 P]ATP and incubated them under identical conditions as used in the RNAi assay shown in Figure 2B. Incubation of dsRNA in the *Drosophila* extract gave rise to siRNAs, while no siRNAs were generated from ssRNA (Figure 2D, compare lanes 1 and 7). In this experiment ~5% of the input dsRNA was processed to siRNAs, while in other experiments up to 15% conversion of input dsRNA was observed (data not shown). A progressive decrease in siRNA production was observed to accompany the increase in editing of the d-dsRNA (lanes 2–6), with only background levels detectable at 43% deamination. The reduced levels of siRNA are sufficient to account for the abolition of RNAi at high levels of deamination.

siRNAs contain a similar amount of inosine as input RNAs

It is possible, at intermediate levels of editing (e.g. 19 % A→I conversion), where siRNA production is reduced only ~5-fold, that later steps in RNAi may also be affected if the siRNAs include A→I modifications. However, it is possible that the *Dicer* ribonuclease involved in processing the dsRNA may discriminate against sequences containing I residues due to the altered structure. We therefore carried out an analysis to determine the inosine content of siRNAs generated from d-dsRNA that contained 18% A→I conversion. dsRNA and d-dsRNA were incubated in *Drosophila* extract for 90 min at 25°C to allow production of siRNAs. The siRNAs and unprocessed RNA from both dsRNA and d-dsRNA were subsequently recovered following denaturing gel electrophoresis. The recovered RNAs were digested with RNase P1 and then subjected to TLC to separate 5'-IMP from 5'-AMP (Figure 3). Prior to incubation in *Drosophila* extract, 18% of the A residues in the input d-dsRNA were converted to I, while the dsRNA contained no I (lanes 5 and 6). The siRNAs produced from d-dsRNA contained 15% A→I conversion (lane 1), only marginally less than the 18% conversion in the full-length d-dsRNA recovered from the same lane of the denaturing gel (lane 2), and the input d-dsRNA (lane 5). No inosine was detectable (<0.8%) in the siRNAs generated from the dsRNA or unprocessed dsRNA (lanes 3 and 4). In contrast, Zamore *et al.* (2000) observed ~3–6% A→I conversion of the dsRNA during their RNAi experiments, but <0.7% in the siRNAs. This is consistent with our observations if a small proportion of their dsRNA were heavily edited and thus unable to generate siRNAs. The reason for the lower level of endogenous ADAR activity in our experiments is not clear. It could be related to the age of the embryos used to prepare the extract (3–6 h as opposed to 0–2 h) or to slight differences in the quantity of extract or duration of the RNAi assay. Our data show that moderately deaminated dsRNA can be processed to siRNAs with no strong discrimination against the generation of siRNAs containing I•U base pairs. A 15% level of A→I conversion corresponds to an average of 0.8 inosines per siRNA, a level at which ~55% of the siRNAs would be expected to contain at least one inosine. If perfect Watson–Crick base-pairing between siRNAs and the targeted RNAs is required, the presence of I in the siRNAs will impair RNAi disproportionately more than can be accounted for by the reduced amounts of siRNA. This could be tested directly by carrying out *in vitro* RNAi experiments with synthetic siRNAs containing single A→U to I•U substitutions.

Fig. 3. siRNAs contain inosine. dsRNA and d-dsRNA (with 18% A→I) were incubated in *Drosophila* extract for 90 min at 25°C. The siRNAs (and unprocessed RNA) generated from both dsRNA and d-dsRNA were recovered following gel electrophoresis and analysed for inosine content by RNase P1 digestion followed by TLC. The input d-dsRNA contained 18% A→I conversion (lane 5), while the dsRNA contained no inosine (lane 6). Following recovery from the gel, the unprocessed dsRNA (L) and siRNA generated from dsRNA contained no inosine (lanes 3 and 4). In contrast, unprocessed d-dsRNA (L) contained an equivalent amount of inosine as in the input d-dsRNA (18% A→I, lane 2). SiRNAs generated from d-dsRNA contained inosine at a level that corresponds to 15% A→I conversion.

Our experiments confirm the predicted biochemical antagonism between ADARs and RNAi (Bass, 2000). We used human ADAR2, but similar results would be expected with other ADARs, which show similar maximal levels of ~50% A→I conversion upon hyper-editing of long dsRNA substrates (Hough and Bass, 2000). Even if there were some differences in the preferences for editing particular adenosines, the overall structural distortions in the dsRNA would be similar, resulting in lack of processing by *Dicer* enzymes. At present, it is not clear what physiological consequences result from this antagonism. Expression of ADARs in various organisms is highest in neural tissues and also in the developing vulva of *C. elegans* (L. Tonkin and B. Bass, personal communication). RNAi in most *C. elegans* tissues can be readily achieved by soaking the worms in dsRNA or feeding with *Escherichia coli* strains that express a trigger dsRNA. In contrast, neurons are relatively resistant (Kamath *et al.*, 2000; Maeda *et al.*, 2001), and efficient RNAi of neuronally expressed genes has only been achieved by *in vivo* expression of a heritable inverted repeat gene corresponding to the target gene under the control of a strong heat shock-inducible promoter (Tavernarakis *et al.*, 2000). Likewise, injection of dsRNA was unable to invoke RNAi in the vulva (Fire *et al.*, 1998). It is tempting to speculate that the high levels of ADARs in neurons and vulva can explain, at least in part, the relative resistance of genes in these tissues to RNAi (Bass, 2000). This should be readily testable with ADAR knockout strains of *C. elegans*.

Both RNAi and ADARs have been suggested to have roles in antiviral defence, although both systems also have roles in normal development (Emeson and Singh, 2000; Hough and Bass, 2000; Grishok *et al.*, 2001; Hutvagner *et al.*, 2001). Mammalian ADARs are usually confined to the nucleus, although an interferon inducible ADAR1 isoform is cytoplasmic (Patterson and Samuel, 1995). The subcellular localization of the RNAi machinery has not been investigated, but cytoplasmic

localization appears likely (Fire *et al.*, 1998). Thus, in some circumstances the two systems may be able to compete directly for access to dsRNA, while elsewhere competition might be determined by which system is first to encounter the dsRNA and thus convert it to a form that cannot be recognized by the other system.

METHODS

RNAs. All RNAs were prepared by *in vitro* transcription. The CAT target RNA and the control RNAs were labelled internally with [α - 32 P]UTP (3000 Ci/mmol; Amersham), where 50 μ Ci [α - 32 P]UTP was added per 50 μ l transcription reaction. The 'unlabelled' trigger RNAs were trace-labelled with [α - 32 P]UTP (3000 Ci/mmol; Amersham) at a specific activity at least 250-fold lower than that of the target RNA. The labelled trigger RNAs were labelled internally (on the sense strand only) with [α - 32 P]ATP (3000 Ci/mmol; Amersham) to a similar specific activity as the target RNAs. All of the RNAs were initiated using an m⁷G(5')ppp(5')G dinucleotide primer. dsRNAs were prepared by annealing complementary RNAs (Scadden and Smith, 2001).

Δ KP RNA (Scadden and Smith, 1997) was synthesized using SP6 RNA polymerase following linearization with *Bam*HI to give a 296-nt transcript. PV 1 RNA (Scadden and Smith, 2001) was synthesized using T7 RNA polymerase following linearization with *Bam*HI to give a 230-nt transcript. CAT target RNA (O'Connell and Keller, 1994) was synthesized using T7 RNA polymerase after linearizing the plasmid with *Pvu*II to give an 858-nt transcript. CAT dsRNA was prepared as described previously (Scadden and Smith, 2001). VLE RNA comprised nucleotides 1383–1813 from the Vg1 cDNA (Pressman-Schwartz *et al.*, 1992).

RNAi assay. The RNAi assay was performed using the conditions described previously (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). The *Drosophila* extract was prepared from 3–6 h PEL embryos according to the method of Becker *et al.* (1994). Electrophoresis on a 15% polyacrylamide gel enabled the analysis of siRNAs. Data were quantitated following phosphorimaging using Imagequant software (Molecular Dynamics).

Deamination reaction. Deamination reactions were carried out as described previously using recombinant human ADAR2 (Scadden and Smith, 2001). The time of the deamination reaction was adjusted to achieve varying levels of editing. Deamination reactions using labelled and unlabelled dsRNAs were carried out in parallel to enable quantitation of unlabelled d-dsRNAs. To analyse the efficiency of the deamination reactions, 50 fmol of RNA were digested with RNase P1 and analysed by TLC (Scadden and Smith, 2001).

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