Biochemical characterization of human Dicer and its interacting partner TRBP

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Summary

Over the recent years, RNA interference (RNAi) has emerged as a powerful method to study the role of individual genes. However, the mechanism underlying the gene silencing by the double-stranded RNA (dsRNA) is still not fully understood. RNAi is initiated when dsRNA is processed by RNase III endonuclease Dicer into short interfering RNAs (siRNAs), of 21 to 22 nucleotides in length. SiRNAs are then incorporated into RNA-induced Silencing Complex (RISC) that by base-pairing targets messenger RNA for degradation. Dicer is also involved in processing of precursors of the small regulatory RNA species, microRNAs (miRNAs). MiRNAs are encoded in the genome and are implicated in gene expression regulation in various cellular processes. After maturation by Dicer, miRNAs are incorporated into RISC-like complexes that in animals imperfectly base-pair with the target mRNA and lead to inhibition of translation. This thesis focuses on Dicer, the central protein involved in both RNAi and miRNA pathways.

Detailed study of the ribonuclease activity of human Dicer and its ancestral prototype, bacterial RNase III, are described in the first experimental chapter of this thesis. The common model for dsRNA cleavage by the RNase III-class enzymes is proposed. The use of mutagenesis to investigate the catalysis revealed that Dicer and bacterial RNase III contain a single compound catalytic center. Both RNase III domains of Dicer contribute to the dsRNA cleavage reaction. The results obtained in this study have proved the then-accepted model of RNase III catalysis to be inadequate. We demonstrated that instead of the two

catalytic centers as proposed in the old model, both *E. coli* RNase III and Dicer contain one compound catalytic center that generates products with 2-nt 3' overhangs. *In silico* modeling of the dsRNA substrate into 3D crystal structure coordinates of the bacterial RNase III offered additional support to our interpretation. Together with other data, a new model was proposed according to which Dicer functions as an intramolecular pseudodimer of its two RNase III domains, assisted by the flanking RNA binding domains, PAZ and dsRBD.

Second chapter describes dsRNA binding domain (dsRBD)-containing protein, TRBP, that was found to associate with Dicer in mammalian cells and *in vitro*. We show that TRBP is required for optimal RNA silencing mediated by siRNAs and endogenous miRNAs, and that it is involved in efficient processing of pre-miRNAs. Since TRBP had previously been described as the inhibitor of the interferon-induced double-stranded RNA-regulated protein kinase PKR, the TRBP-Dicer interaction raises a possibility of the connection between RNAi and interferon-PKR pathways.

DsRNA binding properties of human Dicer dsRBD are described in the third chapter. We have found that this domain has the propensity to bind dsRNAs of different lengths. Surprisingly, it displays hardly detectable affinity for siRNAs. This observation suggests that the dsRBD might be involved in substrate binding during Dicer cleavage reaction and take part in substrate/product discrimination preventing the enzyme from sequestering its own product.

The two supplementary chapters contain the work performed in collaboration with other laboratories. We show that like its *Drosophila* counterpart,

human Dicer is able to form complexes with siRNAs both *in vitro* and *in vivo*. These results indicate that also in mammals Dicer could function downstream of the dsRNA cleavage step and could take part in RISC assembly. The other supplementary chapter describes RNAi connection with chromatoid bodies during spermatogenesis. We show that Dicer and components of the RISC-like complex (Ago and miRNA) are concentrated in chromatoid body. We also demonstrate that Dicer directly interacts with the RNA helicase MVH (mouse Vasa homolog) that is the germ-line specific chromatoid body component. Our findings suggest that the chromatoid body might function as a subcellular concentration site for the miRNA pathway components during spermatogenesis.

INTRODUCTION

RNA interference (RNAi) is a posttranscriptional gene regulatory pathway that is triggered by double-stranded RNA (dsRNA). Since the discovery that introduction of dsRNA into cells initiates sequence specific gene silencing (Fire et al., 1998), the use of RNAi as a laboratory tool has revolutionized the study of eukaryotic gene function. The discovery of RNAi was awarded the Nobel Prize in Physiology or Medicine in 2007 to Andrew Z. Fire and Craig C. Mello.

The discovery of RNAi

RNA interference (RNAi) was discovered through three independent lines of experiments. The most familiar work is that from the laboratories of Fire and Mello. They made the groundbreaking discovery that double-stranded RNA (dsRNA) could induce gene silencing in the nematode *Caenorhabditis elegans* (Fire et al., 1998). Prior to this discovery, however, Baulcombe and coworkers and also other laboratories have described co-suppression or PTGS (Post-transcriptional gene silencing), gene silencing mechanism in plants that was triggered by viral replication or transgene expression (de Haan et al., 1992; English et al., 1996; Hobbs et al., 1993; Lindbo et al., 1993). The short interfering RNA (siRNA) was then first discovered in plant systems (Hamilton and Baulcombe, 1999) and subsequent identification of siRNAs in *Drosophila melanogaster* provided the connection between gene silencing pathways across kingdoms (Hammond et al., 2000; Zamore et al., 2000). The third line of experimentation leading to the discovery of RNAi mechanisms dealt with the the study of developmental timing in *C. elegans*. Laboratories of Ambros and Ruvkun

(Lee et al., 1993; Wightman et al., 1993) had identified lin-4, a small untranslated RNA that was involved in the regulation of expression of the mRNA encoding lin-14. This was the first identified microRNA (miRNA). We now know that miRNAs are naturally occurring molecules that trigger the RNA silencing pathway and play an essential role in gene regulation at the posttranscriptional level in many organisms, including plants, nematodes, flies, and humans.

Mechanism of RNAi

The RNAi machinery consists of a conserved core of factors with roles in recognizing, processing and effecting responses to dsRNA. The general two step model is proposed to describe the mechanism of RNAi (Fig. 1). The first step, RNAi initiation, involves the processing of dsRNA into discrete 21- to 25-nucleotide long (21-25-nt) dsRNA fragments, siRNAs, by ribonuclease (RNase) type III Dicer. Subsequently, siRNAs are involved in RNAi effector step and join a multiprotein complex that base-pairs to mRNAs and leads to their degradation.

The initiation step of RNAi

DsRNA introduced into cells by viral infection, artificial expression or formed in cells by synthesis of complementary strands is processed to ~20-bp siRNAs containing 2-nt 3' overhangs. Proteins involved in siRNA generation include RNase III endonuclease Dicer along with its associated partners: TRBP in mammals (this work; (Chendrimada et al., 2005; Lee et al., 2006), R2D2 and Loquacious in *Drosophila* (Forstemann et al., 2005; Liu et al., 2003; Pham et al.,

2004; Saito et al., 2005), and RDE-4 in *C. elegans* (Grishok et al., 2000; Tabara et al., 1999).

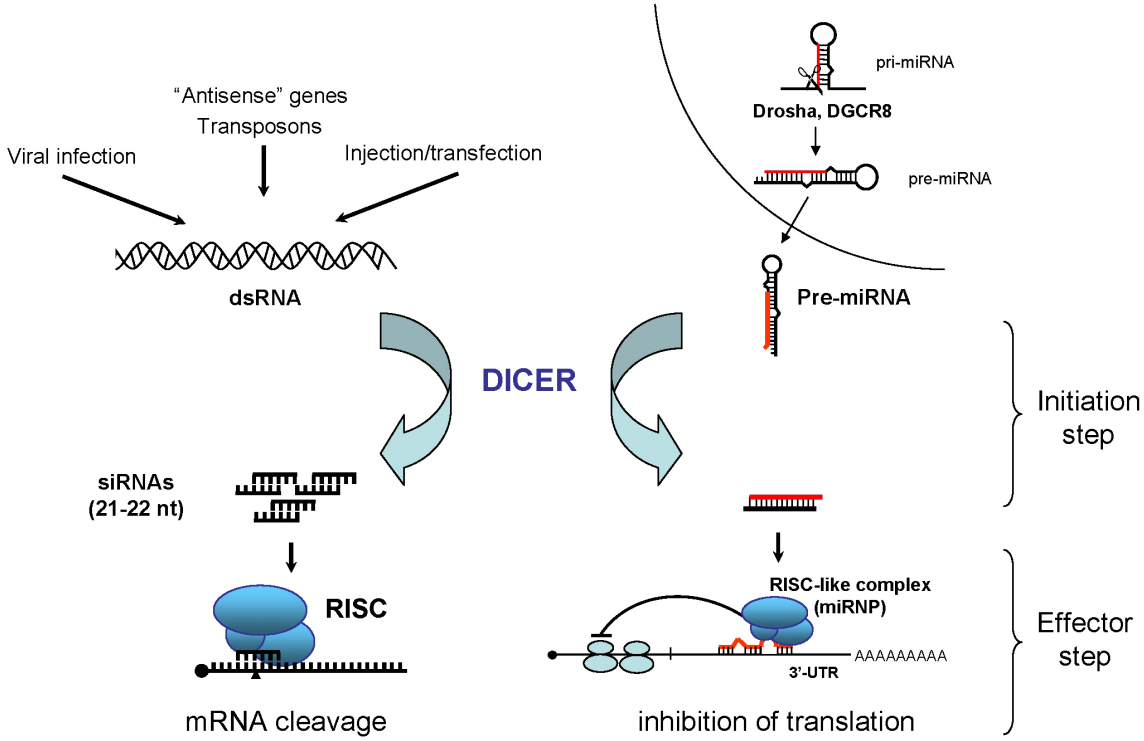

Fig. 1. Scheme of siRNA and miRNA mediated gene silencing. Dicer is the central enzyme involved in both pathways, processing long dsRNAs into siRNAs and pre-miRNAs into mature miRNAs.

The effector step of RNAi

SiRNAs produced by Dicer are handed over to the RNA-induced silencing complex (RISC) where the cognate mRNA is identified by base-pairing and targeted for degradation. Most important component of RISC is a PPD protein family member, Argonaute, that cleaves mRNA in the middle of siRNA-mRNA complementarity (Liu et al., 2004; Meister et al., 2004). Other factors cofractionating with RISC include the Vasa intronic gene (VIG) protein, Tudor-SN

nuclease and dFXR, the *Drosophila* ortholog of human fragile X mental retardation protein (FMRP) (Caudy et al., 2003; Caudy et al., 2002; Ishizuka et al., 2002).

Gene regulation by miRNAs

RNAi machinery is also involved in the miRNA-mediated gene regulation pathway. MicroRNAs are a family of small, non-coding RNAs that regulate gene expression in a sequence-specific manner (reviewed by (Bartel, 2004). MiRNAs are generated from the genome-encoded precursor hairpins (Lagos-Quintana et al., 2001) by the sequential action of two RNase III type nucleases, Drosha in the nucleus (Lee et al., 2003) and Dicer in the cytoplasm (Bernstein et al., 2001).

Discovery of miRNAs

The founding member of the miRNA family, lin-4, was discovered in nematode *C. elegans* through a genetic screen for defects in the temporal control of post-embryonic development. Mutations in lin-4 disrupt the temporal regulation of larval development, causing the first larval stage-specific cell-division patterns to reiterate at later developmental stages (Chalfie et al., 1981). Opposite developmental phenotypes are observed in worms that are deficient for lin-14 (Ambros and Horvitz, 1984). Most genes identified from mutagenesis screens encode proteins, but lin-4 encodes a 22-nucleotide non-coding RNA that is partially complementary to 7 conserved sites located in the 3'-untranslated region (3'-UTR) of the lin-14 gene (Lee et al., 1993; Wightman et al., 1993). Lin-14

encodes a nuclear protein that has to be downregulated at the end of the first larval stage to promote the developmental progression into the second larval stage. Both, the functional lin-4 gene and an intact 3'-UTR of its mRNA, are essential for the negative regulation of LIN-14 protein expression. It has been demonstrated that the imperfect base pairing between lin-4 and the lin-14 3'-UTR was essential for the ability of lin-4 to control LIN-14 expression at the level of protein synthesis (Ha et al., 1996; Olsen and Ambros, 1999).

The discovery of lin-4 and its target-specific translational inhibition pointed at a new mechanism of gene regulation during development. Second miRNA, let-7 was identified, hinting that lin-4-type regulation of gene expression was not an isolated case but possibly a general mechanism. Let-7 encodes a temporally regulated 21-nucleotide small RNA that controls the developmental transition from the last larval stage into the adult stage (Reinhart et al., 2000). Similar to lin-4, let-7 imperfectly binds to the 3'-UTR of lin-41 and hbl-1 mRNAs and inhibits their translation (Abrahante et al., 2003; Lin et al., 2003).

Identification of let-7 raised the possibility that similar RNAs might be present in species other than nematodes. Both let-7 and lin-41 are evolutionarily conserved throughout metazoans, with homologues that were detected in mollusks, sea urchins, flies, mice and humans (Pasquinelli et al., 2000). This extensive conservation strongly indicated a more general role of small RNAs in developmental regulation. Orthologues of lin-4 were also later identified in flies and mammals (Lagos-Quintana et al., 2002; Sempere et al., 2002).

Hundreds of miRNAs have now been identified in various organisms (Bartel and Chen, 2004), and the RNA structure and regulatory mechanisms that have been characterized in lin-4 and let-7 define unique molecular signatures defining miRNAs. MiRNAs are generally 21–25 nucleotide-long, non-coding RNAs that are derived from larger, genome encoded precursors transcribed by RNA polymerase II (Pol II), forming partially double stranded stem-loop structures (reviewed by (Bartel, 2004). The mature miRNA is most often derived from one arm of the precursor hairpin, and is released from the primary transcript through stepwise processing by two RNase III enzymes. In animals, most miRNAs bind with imperfect complementarity to the target mRNA 3'-UTR and function as translational repressors.

SiRNA and miRNA pathways

Both, siRNAs and miRNAs, can silence cytoplasmic mRNAs either by triggering a endonucleolytic cleavage or by promoting repression of translation. A key difference between siRNA and miRNA function is the specificity of their interactions with the target mRNA. Generally, siRNA base-pair perfectly to the mRNA and trigger the endonucleolytic cleavage between bases 10 and 11 of the duplex (Elbashir et al., 2001; Haley and Zamore, 2004; Hammond et al., 2000; Martinez and Tuschl, 2004; Tuschl et al., 1999). In plants, also most of the miRNAs direct endonucleolytic cleavage (Llave et al., 2002). In metazoan, there are also some examples of perfect base-pairing between endogenous miRNA and mRNA leading to the mRNA cleavage (Yekta et al., 2004). However, most

metazoan miRNA form imperfect mismatches and trigger translational repression (reviewed by (He and Hannon, 2004). From a number of experiments, some key principles the miRNA/target interaction have emerged. Base-pairing between the 5' end of the miRNA (residues 2–7, a 'seeding region') and the mRNA target plays a primary role in establishing the interaction (Doench and Sharp, 2004). Moreover, the 5' portion of related miRNAs is the most highly conserved. The 3' portion of the miRNA contributes to efficient repression, and it has been suggested to work as a modulator of suppression (Doench and Sharp, 2004; Kiriakidou et al., 2004; Kloosterman et al., 2004).

While only one complementary site is generally sufficient to direct repression by cleavage, with a few exceptions multiple sites are required for efficient translational repression (Doench et al., 2003; Doench and Sharp, 2004; Kiriakidou et al., 2004; Zeng et al., 2003). In addition, the binding of the miRNP to the mRNA might be influenced by other RNA-binding proteins such as GW182 that interacts with Argonaute proteins and is required for efficient miRNA-mediated repression in animals (Jakymiw et al., 2005; Liu et al., 2005a; Rehwinkel et al., 2005).

Some recent data suggest that miRNAs downregulate translation at the initiation step and that repressed mRNAs are subsequently relocalized to P-bodies (processing bodies) that contain pools of mRNAs not engaged in translation or undergoing degradation (Brengues et al., 2005; Kedersha et al., 2005). Ago proteins, miRNAs, and repressed mRNA targets are enriched in P-bodies (Jakymiw et al., 2005; Liu et al., 2005b; Pillai et al., 2005; Sen and Blau,

2005). Some other observations suggest that miRNAs might also repress translation by affecting a step in protein production after translation initiation, possibly causing ribosome drop off during elongation of translation (Petersen et al., 2006). The possibility that miRNAs and RISC can drive translation repression by multiple mechanisms cannot be excluded at present.

Ribonuclease III superfamily

The discovery that RNase III enzymes are involved in RNAi and miRNA pathways in various organisms resulted in renewed interest in this class of enzymes. A classification scheme for RNase III orthologs has been proposed (Blaszczyk et al., 2001) (Fig. 2). Class I, including eubacterial enzymes and the yeast ortholog Rnt1p, has the simplest domain organization, with a single catalytic domain and a dsRNA binding domain (dsRBD). Rnt1p has an N-terminal extension that was shown to be important for dimerization activity of the enzyme (Lamontagne et al., 2000). Class II enzymes, including Drosha proteins, have a duplicated catalytic domain, a single dsRBD, and a variable length N-terminal region with the proline-rich and arginine/serine-rich domains. Middle part, lacking a distinguishable motif, is needed for interaction with a partner protein, such as, for example, DGCR8 (Han et al., 2004). Drosha enzymes are found only in animals. Class III includes Dicer homologs containing two catalytic domains, dsRBD, DUF283 (domain of unknown function), and PAZ (Piwi/Argonaute/Zwille) and ATPase/helicase domains. Dicers are found in most eukaryotes (e.g. *Schizosaccharomyces pombe*, plants and animals. Dicer gene is not present in

Saccharomyces cerevisiae. Enzymes of RNase III class are not present in archaea.

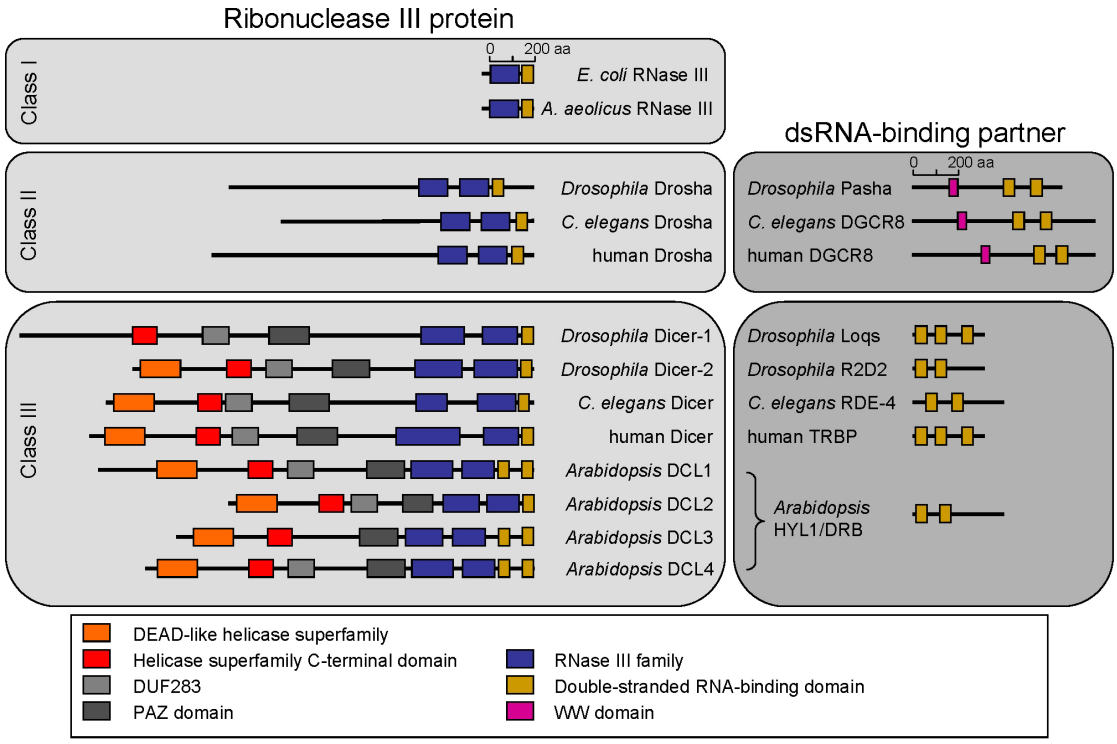

Fig. 2. Classification of RNase III orthologs and their associated dsRBD-containing partners. Individual protein domains are depicted as colored blocks.

Function of RNase III

RNase III was first discovered as a dsRNA degrading activity in *Escherichia coli*. RNase III autoregulates its own expression by cleavage of a stem-loop upstream of its ORF (Bardwell et al., 1989). Bacterial RNase III can be phosphorylated by a phage T7 encoded serine–threonine protein kinase that leads to an increase of the dsRNA cleavage activity (Mayer and Schweiger, 1983).

The main function of RNase III is in pre-rRNA processing. In addition, bacterial RNase III participates in the maturation of mRNAs, excising mRNAs and co-transcribed tRNAs or intercistronic regions from polycistronic mRNA precursors. RNase III has a role in mRNA turn-over by cleaving mRNAs within 5'-untranslated regions (5'-UTRs) (Regnier and Grunberg-Manago, 1990). Maturation of tRNA precursors or processing of phage and plasmid transcripts is often dependent on RNase III (reviewed by (Nicholson, 1999). It has been proposed, that RNase III can also exert regulatory functions within the cell through binding of a particular RNA, without its cleavage (Dasgupta et al., 1998). Although RNase III is involved in many important cellular processes, its deletion is not lethal but just results in a slower growth phenotype of *E. coli* (Babitzke et al., 1993) which suggests that some of the reactions catalyzed by RNase III can be carried out by other RNases. However, in *Bacillus subtilis* and in the 'minimal' genome of *Mycoplasma genitalium,* RNase III gene is essential (Herskovitz and Bechhofer, 2000; Hutchison et al., 1999).

In eukaryotes, the first studied RNase III enzymes were the *S. cerevisiae* Rnt1p and *S. pombe* Pac1p. Both are essential for viability (Abou Elela and Ares, 1998; Chanfreau et al., 1998). The yeast RNases III have an N-terminal extension which at least in the case of the *S. cerevisiae* enzyme facilitates dimerization of the protein (Lamontagne et al., 2000). In eukaryotes, RNase III is involved in rRNA maturation by cleaving pre-rRNA in 3'-ETS (external transcribed spacer) (Kufel et al., 1999) and processing of some small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) (Chanfreau et al., 1998; Qu

et al., 1999; Seipelt et al., 1999). However, RNase III is not a part of the yeast exosome complex which also processes sn(o)RNAs (Mitchell and Tollervey, 2000).

Endonucleolytic cleavage by RNase III creates dsRNA fragments with 5'-phosphates and 3'-OH groups containing 2 nt 3'-overhangs. When perfectly base-paired dsRNA substrates such as poly I-C RNA are incubated with RNase III, exhaustive digestion yields dsRNA fragments of 12–15 nt in lenght. The minimum substrate length is ~20 base-pairs (bp), equivalent to about two turns of an A-form dsRNA. The cleavage site selection appears to be random (reviewed by (Nicholson, 1996). In imperfect duplexes RNase III specificity is not determined by a clearly defined sequence motives within the substrate, as in the case of type II restriction endonucleases. Physiological RNase III substrates are cleaved at exactly defined positions, although they lack a consensus motif. Current model for cleavage site determination by the *E. coli* RNase III is based on the presence or absence of so-called anti-determinants, nucleotides that have to be present or absent in certain regions (distal and proximal boxes, respectively) close to the cleavage site (Zhang and Nicholson, 1997). This model, however, is not universally valid for all RNase III substrates (Evguenieva-Hackenberg and Klug, 2000).

Catalysis by RNase III possibly follows a two metal ion mechanism. Consistently, in order to cleave its substrate RNase III needs divalent cations, preferably magnesium. Magnesium ions can be substituted for by manganese, cobalt or nickel ions, but these can result in altered cleavage specificity.

Recognition and binding of the RNA is possible in the absence of divalent metal ions (Li et al., 1993).

Dicer

Dicers are approximately 200 kDa multidomain proteins. The PAZ, dsRBD and RNase III domains are involved in dsRNA binding and cleavage. The PAZ domain is also found in PPD (PAZ and Piwi Domain) or Argonaute proteins that interact with Dicer and are involved in RNAi and miRNA function. Structural studies of the PAZ domain of the *Drosophila* PPD proteins Ago-1 and Ago-2 revealed a similarity to the oligonucleotide binding (OB) fold, consistent with the RNA binding activity of the domain (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). The presence of the helicase/ATPase domain could be attributed to the observation that generation of siRNAs by the *C. elegans* Dicer and one of the two *Drosophila* Dicers seems to be of ATP-dependant (Bernstein et al., 2001; Ketting et al., 2001; Liu et al., 2003; Nykanen et al., 2001). However, no such effect is observed for the mammalian enzyme (Zhang et al., 2002). Moreover, Dicers of *Dictyostelium* and *Giardia intestinalis* are devoid of the helicase/ATPase domain (Macrae et al., 2006; Martens et al., 2002).

Mammalian genome encodes one Dicer gene. Plants, such as *Arabidopsis thaliana*, poplar and rice express four dicer-like proteins (Dcl). Fungi, such as *Neurospora crassa* and insects, such as *Drosophila,* and mosquito, contain two Dicer genes. Four plant Dicer-like proteins have distinct roles: Dcl-1 processess miRNAs, Dcl-2 generates siRNAs associated with virus defense,

Dcl-3 produces siRNAs that are involved in chromatin modification and transcriptional silencing, and Dcl-4 creates trans-acting siRNAs (tasiRNAs) that regulate vegetative phase change (Borsani et al., 2005; Gasciolli et al., 2005; Park et al., 2002; Xie et al., 2005; Xie et al., 2004). Since the small RNAs produced by different Dcls are involved in diverse processes, there must be a mechanism that allows efficient substrate RNA discrimination and subsequent incorporation of the product into correct effector complexes. It has been suggested that dsRBD might be involved in mediating this process. Dcls, with the exception of Dcl-2, contain two dsRBDs, while Dcl-2 contains one such domain. It has been noted that the variation between the pair-wise sequence alignments of Dcl-types 1, 3 and 4 is most pronounced in this domain, what might account for potentially different substrate specificity (Margis et al., 2006). Fusion proteins containing both dsRBD1 and dsRBD2 domains of Dcl-1, Dcl-3 and Dcl-4 can bind to members of the HYL1/DRB family of proteins that are implicated in small RNA pathways in *Arabidopsis* (Hiraguri et al., 2005). The model has been proposed that the dsRBD domain along with the PAZ, and RNase IIIa and b domains recognizes and processes specific RNA substrates and, by specific interaction with different HYL1/DRB members, directs the newly generated small RNAs to their appropriate effector complexes (Margis et al., 2006).

Studies in *Drosophila* established distinct roles for the two *Drosophila* Dicer proteins. Dicer-1 is essential for miRNA processing while Dicer-2 is necessary for siRNA production. Separation of function for *Drosophila* Dicers is not absolute. Although Dicer-1 and Dicer-2 generate different types of small

RNAs, both are required for siRNA-directed target mRNA cleavage and gene silencing. Dicer-2 is required to form a stable siRNA-protein complex that initiates siRISC assembly and contains Dicer-2 and R2D2 (Lee et al., 2004b; Liu et al., 2003; Pham et al., 2004). Dicer-1 is required for correct assembly of the intermediate complex from its precursor initiator complex (Lee et al., 2004b).

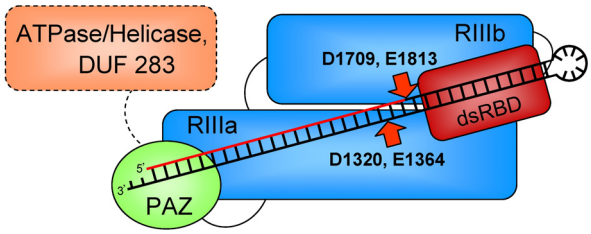

Fig. 3. A model of pre-miRNA processing by Dicer. Individual domains of Dicer shown are in different colors. The enzyme contains a single dsRNA cleavage center with two independent catalytic sites. The center is formed by the RIIIa and RIIIb domains of the same Dicer molecule. The placement of the RIIIa domain illustrates the fact that this domain cleaves the 3'-OH-bearing RNA strand. DsRBD is arbitrarily positioned on the substrate.

dsRNA and pre-miRNA processing by Dicer

In the course of this work the mechanism of Dicer cleavage of dsRNA and pre-miRNA was investigated and a model of the cleavage has been proposed (Fig. 3). Briefly, Dicer works as an intramolecular pseudodimer with RNase IIIa and IIIb forming a single catalytic center containing two independent catalytic 'half sites', each capable of cutting one RNA strand of the duplex to generate products with 2-nt 3' overhangs. The end of the dsRNA substrate is recognized by the PAZ domain. A model proposed in this work is strengthened by the recent determination of the crystal structure of the full-length *Giardia intestinalis* Dicer

(Macrae et al., 2006). The RNase III domains form the catalytic domain and the PAZ domain is directly connected to the RNase IIIa domain by a long α-helix dubbed the 'connector' helix which is implicated in determining the product length (Fig. 4).

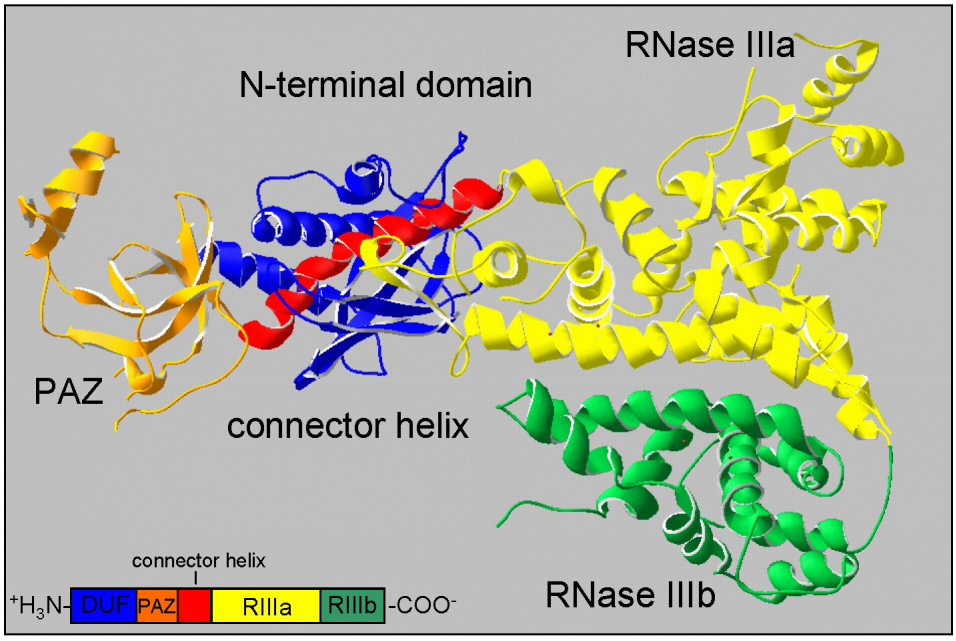

Fig. 4. Crystal structure of Giardia Dicer. Ribbon representation of Dicer shows the N-terminal platform domain (blue), the PAZ domain (orange), the connector helix (red), the RNase IIIa domain (yellow), the RNase IIIb domain (green).

From the crystal structure the role for conserved 'domain of unknown function 283' (DUF283) was proposed. Low but significant sequence homology between the N-terminal domain of *Giardia* Dicer and DUF283 suggests that DUF283 forms a platform structure similar to that of the *Giardia* Dicer also in the Dicers of higher eukaryotes. Based on computational modeling it was proposed that DUF283 could possibly adapt a dsRBD fold (Dlakic, 2006). It is worth noting that although *Giardia* Dicer contains neither helicase nor dsRBD domains found

in other Dicers, it is capable of complementing the Dicer deletion mutant of fission yeast.

After completing the cleavage reaction Dicer remains associated with the product (Zhang et al., 2002). This observation is consistent with the involvement of Dicer in the effector step of RNAi. For the siRNAs to act as a guide for mRNA cleavage, it must be unwound into its component strands and then reassembled with proteins to form active RISC. The strand that is going to be incorporated into RISC is called the guide strand, while the second passenger strand undergoes degradation. Strand selection mechanism exists to ensure an effective and efficient siRNA loading to RISC. Thermodynamic differences in the base-pairing stabilities of the 5' ends of the two siRNA strands determine which strand is assembled into the RISC (Khvorova et al., 2003; Schwarz et al., 2003). In *Drosophila*, the strand selection is achieved by appropriate orientation of the Dicer-2/R2D2 heterodimer on the siRNA duplex. R2D2 binds the siRNA end with stronger thermodynamic stability, Dicer binds the opposite less stable end, and with the strand with its 5'-terminus at this end is selected as a guide and becomes a part of an active RISC (Preall et al., 2006; Tomari et al., 2004b). In addition, guide strand selection does not depend on Dicer processing (Preall et al., 2006). Both partners, Dicer and R2D2 act as a protein sensor for determination of siRNA thermodynamic asymmetry. Dicer always approaches the substrate from the end (Zhang et al., 2002) and processes it in a polar way with RNase IIIa domain cleaving 3'-hydroxyl and RNase IIIb cleaving the 5'-phosphate-bearing RNA strand (this work). Hence, the asymmetry of the short

RNA duplex at least in some cases has to be determined not at the level of cleavage, but afterwards. When siRNA generated by Dicer happens to have a thermodynamically unfavorable end associated with the enzyme, it needs to be released from Dicer after the cleavage, and then re-bound by the Dicer/R2D2 heterodimer. The fact that the siRNA strand decision is not random but follows well defined thermodynamic rule argues in favor of existence of such an siRNA 'flipping'; however, the mechanism underlying the process is not known. Similar strand choosing mechanism has to be employed for the correct selection of a mature miRNA, as miRNA originate from either ascending or descending strand of the pre-miRNA hairpin. Like siRNAs, miRNAs show polarity in their reactivity and thermodynamic stability that define the active strand which encodes mature miRNA sequence (Krol et al., 2004).

dsRBD-containing cofactors of Dicer

During cleavage of their substrates Dicer and Drosha function as components of larger complexes. They seem to invariably associate with dsRBD-domain-containing protein cofactors (Fig.2). The first dsRBD protein, Rde-4 (RNAi deficient-4), was identified in a genetic screen in *C. elegans* (Tabara et al., 1999). It is required for the initiation step of RNAi in worms, but its activity is not required for miRNA processing or worm development (Grishok et al., 2000). In *Drosophila*, Dicer-1, Dicer-2 and Drosha are associated with Loquacious (Loqs), R2D2, and Pasha, respectively (Denli et al., 2004; Forstemann et al., 2005; Gregory et al., 2004; Landthaler, 2004; Liu et al., 2003; Saito et al., 2005). The role of R2D2 in

directing strand specific incorporation of the siRNA is well established. Heterodimer of Dicer-2 and R2D2 senses the stability of the duplex ends and determines which strand will enter the RISC. Photocrosslinking to siRNAs containing 5-iodouracils revealed that Dicer binds to the less stable and R2D2 to the more stable siRNA end (Tomari et al., 2004b). Since the siRNA asymmetry rules are quite similar in all organisms, R2D2-related proteins could likely be involved in the definition of siRNA strands in other organisms. Loqs associates with Dicer-1 and is required for providing the correct substrate specificity for pre-miRNAs to Dicer-1 (Saito et al., 2005) and enhanced processing activity (Forstemann et al., 2005; Saito et al., 2005)

In humans, Drosha associates with DGCR8 (Han et al., 2004) and in the course of this work TRBP [human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein] was found to be a dsRBD protein partner of human Dicer. TRBP is required for optimal RNA silencing mediated by siRNAs and endogenous miRNAs. However although related to R2D2/Loqs, TRBP was not to date demonstrated to be involved in definition of siRNA asymmetry. TRBP has previously been assigned several functions, including inhibition of the interferon (IFN)-induced dsRNA regulated protein kinase PKR (Daher et al., 2001), modulation of HIV-1 gene expression through its association with TAR (Dorin et al., 2003), and control of cell growth (Benkirane et al., 1997; Lee et al., 2004a). A mouse TRBP homologue, Prbp, was shown to function as a translational regulator during spermatogenesis, and mice that have its deletion were male sterile and usually died at the time of weaning (Zhong et al., 1999).

Argonautes / PPD proteins

The genes encoding *Arabidopsis thaliana* AGO1 and ZWILLE were the first PPD family members to be characterized (Bohmert et al., 1998; Moussian et al., 1998). Before the discovery that PPD proteins are RNAi effectors, AGO1 and ZWILLE were shown to have overlapping functions in plant development (Lynn et al., 1999). Although ZWILLE has no RNAi-related functions, it is well-documented that AGO1 is important for gene silencing (Boutet et al., 2003; Vaucheret et al., 2004). The *C. elegans* genome encodes total of 27 PPD proteins, mice and humans each contain seven and eight PPD genes, respectively. It appears that PPD proteins evolved to perform highly specialized functions. For example, the PPD proteins RDE-1 and PPW-1 are required for efficient siRNA-mediated mRNA cleavage (Fagard et al., 2000; Tabara et al., 1999; Tijsterman et al., 2002), whereas ALG-1 and ALG-2 are not required for this process. However, ALG-1 and ALG-2 function in maturation and translational inhibition activities of miRNAs that regulate developmental timing pathways (Grishok et al., 2001). In *Drosophila*, Ago-2 is required for the incorporation of siRNAs into RISC and subsequent targeting of cognate mRNAs for destruction. Fly embryos lacking Ago-2 activity are defective for siRNA-targeted mRNA cleavage. In contrast, Ago-1 is required for miRNA biogenesis, but not siRNA-mediated RISC activities (Okamura et al., 2004). In humans, four PPD proteins (Ago-1 through Ago-4) were shown to bind miRNAs, but only Ago-2 is associated with the catalytic activity required for the siRNA guided mRNA cleavage (Liu et al., 2004; Meister et al., 2004).

Structural studies of Ago proteins and their subdomains provided important insights into understanding the mechanism of the effector step of RNAi. X-ray and NMR studies of Argonaute PAZ domains, both free and complexed with RNA, have revealed the similarity to the OB fold and determined that the domain specifically recognizes the 2 nt 3' overhang of the duplex or the 3'-OH end of a single-stranded RNA (Lingel et al., 2004; Liu et al., 2004; Ma et al., 2004). In the structure of the human Ago-1 PAZ, the 2 nt overhang is inserted into a pocket constituted of conserved aromatic and hydrophobic amino acids. In the proximal dsRNA region, only the strand with the anchored protruding 3' terminus is in contact with basic amino acid residues, suggesting that this strand will be retained and will function as mRNA antisense guide after siRNA unfolding (Ma et al., 2004). Although the structures of full-length eukaryotic Ago proteins are not available, important information has been obtained from three-dimensional structures of Ago-like proteins. The Ago-like proteins are encoded in the genomes of some archaea and eubacteria, and their function is not clear.

Crystallization efforts yielded the determination of the structure of PfAgo from *Pyrococcus furiosus* (Song et al., 2004) and AfPiwi from *Achaeoglobus fulgidus* (Parker et al., 2004). The ~85 kDa PfAgo includes both PAZ and PIWI domains. AfPiwi is approximately half the size of PfAgo and structurally corresponds to PfAgo middle and PIWI domains; in the AfPiwi structure, they are referred to as domains A and B and constitute the PIWI fold. PIWI domain of PfAgo and AfPiwi bears striking similarity to RNase H, an enzyme that cleaves the RNA strand in DNA-RNA hybrids (Fig. 5). This suggested that Ago proteins

containing the PIWI domain could be responsible for 'Slicer' activity, performing the siRNA directed endonucleolytic cleavage of mRNA in RISC. RNase H contains a triad of conserved acidic amino acids, DDE, essential for catalysis. A related set of residues, DDH, is conserved in PfAgo and some eukaryotic Argonaute proteins, like Ago-2. Mutagenesis of human Ago-2 demonstrated that all three DDH triad amino acids are involved in mRNA cleavage within RISC (Liu et al., 2004; Rivas et al., 2005). The demonstration that human Ago-2 expressed and purified from *E. coli* is able to cleave target mRNA targeted by a complementary single-stranded siRNA provided the ultimate proof that Ago-2 acts as a Slicer in RISC (Rivas et al., 2005).

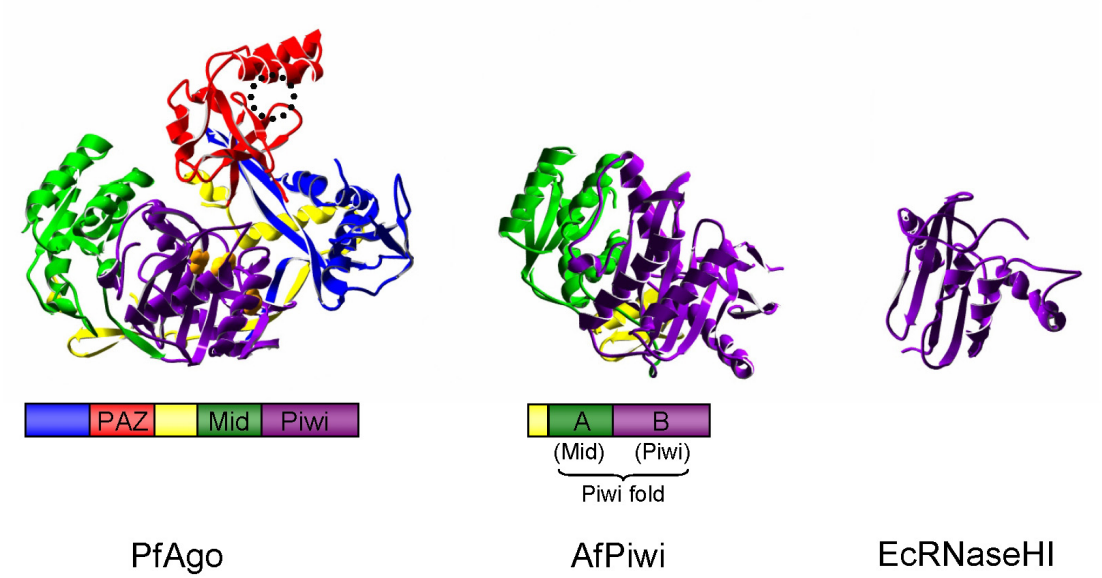

Fig. 5. Structures of PfAgo, AfPiwi and *E. coli* RNase HI shown in a similar view. Structurally conserved domains are traced in the same color and a schematic is shown below. In the PfAgo structure, the DDE triad amino acids are represented as orange balls; a pocket containing conserved amino acids involved in the binding of the 30-protruding nucleotides is marked as a dotted circle.

Several important characteristic features of siRNAs and miRNAs can be explained by the structure of AfPiwi complexed to siRNA-like molecule (Ma et al.,

2005; Parker et al., 2005). It was found both experimentally and bioinfomatically that the terminal 5' nucleotide of miRNA and siRNA does not need to form base pair interaction with the target mRNA (Saxena et al., 2003). In the crystal structure, the 5' phosphate interacts with four conserved basic amino acids and a bound divalent metal ion. Binding of the 5' base is stabilized by stacking on the aromatic ring of conserved tyrosine and the anchored 5' nucleotide is not base paired to the complementary strand, in contrast to the downstream nucleotides, which are engaged in an A-form helix positioned in the basic channel at the A-B domain interface. The observation that the 5' part of miRNA (miRNA 'seed' sequence) has to form a near perfect double stranded duplex with its complementary mRNA sequence to result in efficient silencing is explained by the involvement of the sugar phosphate backbone of four 5'-proximal nucleotides (positions 2 to 5) of the guide strand in contacts with the PIWI domain. The guide nucleotides 2–5 form a quasihelical structure on the PIWI surface that is suitable for base pairing. Modeling of longer A-form helices into the AfPiwi structure placed the scissile phosphate of the mRNA target in the proximity of the proposed catalytic region. This suggests that the mRNA cleavage site is determined by measuring the fixed distance from the anchored siRNA 5' end.

Other proteins identified as RISC components

A number of other factors have been found to associate with Ago proteins in RISC complexes. In *Drosophila* S2 cells, RISCs additionally contain Vasa intronic gene (VIG), dFXR, a *Drosophila* homolog of fragile X mental retardation protein

(FMRP) (Caudy et al., 2002; Ishizuka et al., 2002; Mourelatos et al., 2002) and a protein containing Tudor and staphylococcal nuclease domains (Tudor-SN) that was shown to bind and possibly degrade dsRNAs hyperedited by adenosine deaminases (ADARs), providing a hint of a possible connection between editing and RNAi pathways (Scadden, 2005). A similar complex, containing Argonaute, Tudor-SN and VIG homologs along with siRNAs, was detected in *C. elegans* extracts and mammalian cells (Caudy et al., 2003). Mammalian Argonaute-containing complexes have been found to co-immunoprecipitate with SMN complex proteins Gemin-3 and Gemin-4 (Mourelatos et al., 2002), human FMRP (Hammond et al., 2001), putative RNA helicase MOV10 and the RNA recognition motif (RRM)-containing protein TNRC6B (Meister et al., 2005). The precise function of these proteins in RNA silencing is unknown, however, MOV10 and TNRC6B colocalize with Ago proteins in P-bodies and are required for miRNA-guided mRNA cleavage in cells (Meister et al., 2005).

RISC assembly

The dynamics of RISC formation has been studied by the the application of native gel electrophoresis (Pham et al., 2004; Tomari et al., 2004a) and led to the identification of three stages of RISC formation defined by three distinct siRNA containing complexes: R1, R2 and R3. The R1 complex corresponds to the 360 kDa RISC-like structure described previously (Nykanen et al., 2001). It consists of Dicer-2, R2D2, and one or more yet unidentified proteins. The function of R1 may be the processing of long dsRNA and possibly determination of

guide/passenger strand asymmetry of siRNA. R1 serves as a precursor to R2 and R3 (Pham et al., 2004). R2 is formed with a high rate, which suggests that it may be derived from the binding of R1 to an as yet unidentified pre-assembled complex. The R2 complex is thought to function in siRNA duplex unwinding. The Dicer-2–R2D2 complex senses the thermodynamic stabilities of the ends of the siRNA duplex and selects the guide strand that becomes associated with Ago-2. As siRNA unwinding continues, the Dicer-2–R2D2 complex is replaced with Ago-2, which binds the 2 nt 3' overhang of the guide strand. The unwinding of the siRNA is initiated by the Dicer-2–R2D2 complex, but can proceed only in the presence of Ago-2 (Tomari et al., 2004b). An ATP-dependent DEA(H/D)-box helicase Armitage and PPD protein Aubergine have been implicated in the unwinding process (Cook et al., 2004; Tomari et al., 2004a). The 80S R3 complex whose formation is enhanced by ATP, contains siRNAs, Dicer-1, Dicer-2, VIG, Tudor-SN, Ago-2, dFXR and R2D2. The R3 complex co-purifies with rRNA from small and large ribosomal subunits, suggesting that it is ribosome-associated. R3, the RNAi effector complex has been dubbed a 'holoenzyme'. It may conatain regulatory factors that are not absolutely necessary for mRNA cleavage in vitro (Pham et al., 2004).

The dynamics of RISC assembly in mammals is not as well understood as in *Drosophila*. One of the intermediates has been named complex D, it contains Dicer that is directly bound to siRNA. Complex D might be an equivalent of *Drosophila* R1 complex based on its estimated size of 250-300 kDa (Pellino et al., 2005). Another study argues that human cells contain already preassembled

complex of Dicer, TRBP and Ago-2 capable of binding siRNA duplexes (Chendrimada et al., 2005) and that the complex containing these three components in able to determine the asymmetry of the siRNA duplex and tocorrectly incorporate the guide strand for mRNA cleavage (Gregory et al., 2005). Other data indicate that Dicer might be redundant for the active RISC formation in mammals. HeLa cell extracts immuno-depleted of Dicer retain full siRNA-mediated RISC activity (Martinez et al., 2002) and Dicer-null mouse embryonic stem cells are capable of siRNA-triggered RISC activity (Kanellopoulou et al., 2005). Dicer might play a role in enhancing RISC assembly and function. DsRNAs acting as Dicer substrates are more efficient than siRNA at triggering RISC activity in human cells (Kim et al., 2005; Rose et al., 2005; Siolas et al., 2005), consistent with the possibility that Dicer processing might stimulate RISC assembly.

DsRNA binding domain (dsRBD)

DsRBD has been identified in proteins found in all kingdoms. The functions of dsRBD-containing proteins are diverse and include RNA editing (ADAR1 and ADAR2) (Bass, 1997; Higuchi et al., 2000; Wang et al., 2000), RNA localization and translational control (Staufen) (Ferrandon et al., 1994; Micklem et al., 2000), viral defense and apoptosis (PKR) (Clemens, 1997; Tan and Katze, 1999; Williams, 1999), translational repression (PKR, TRBP, PACT) (Bennett et al., 2004; Gatignol et al., 1991; Gupta et al., 2003), hnRNA association (XLRBPA) (Benkirane et al., 1997), and finally RNA interference (Dicer, RDE-4, R2D2,

Loquacious, TRBP, PACT). The most evident function of the dsRBD is dsRNA binding, but other roles of this domain have been established. The third dsRBD of human ADAR-1 acts as a Nuclear Localization Signal (NLS) and the NLS activity of dsRBD3 does not depend on RNA binding (Eckmann et al., 2001). DsRBD is also involved in mediating protein-protein interactions as it has been shown for TRBP and PACT that their third dsRBD is important for formation of homodimers (Daher et al., 2001).

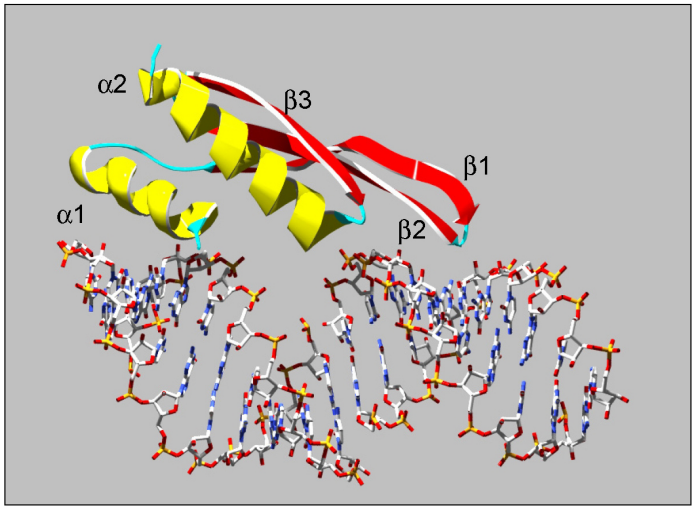

Fig. 6. Structure of the dsRBD2 of XLRBPA bound to a dsRNA helix. Loop between β1 and β2 interacts with the RNA minor groove and loop between β2 and α2 with the major groove. Helix α1 forms another minor groove interaction.

Until now structures of seven dsRBDs have been determined. They include dsRBDs of PKR, XLRBPA, TRBP, Staufen, Rnt1p and RNase III from *Aquifex aeolicus* and *Thermotoga maritima* (Blaszczyk et al., 2004; Nanduri et al., 1998; Ramos et al., 2000; Ryter and Schultz, 1998); PDB 1o0w). In three cases, the second XLRBPA domain, the third Staufen domain and *Aquifex* RNase III, the domain structure was determined in the presence of a bound RNA ligand,

providing insights into the recognition mechanism. The overall structures of the folds are remarkably similar, with three areas of the α-β-β-β-α protein fold lined up on one surface and involved in recognition of the RNA (Fig. 6). Loop 2 interacts with the RNA minor groove and loop 4 with the major groove. Helix α1 forms another minor groove interaction in case of XLRBPA and most interestingly, in the case of Staufen, interacts with a tetraloop present in the hairpin ligand. The XLRBPA thus spans two successive minor grooves and the intervening major groove, covering 16 bp. Loops 2 and 4 change conformation upon RNA binding. The case of tetraloop recognition by Rnt1p dsRBD is particularly interesting, since it resembles the recognition pattern required in sn/sno RNAs and the rRNA 3'-ETS (Chanfreau et al., 2000; Nagel and Ares, 2000). The interactions with the tetraloop are not sequence-specific and nucleotide substitutions have mostly kinetic effects. Although dsRBD structures show how major and minor groove interactions and interactions involving the RNA-specific 2'-OH group discriminate against dsDNA or DNA/RNA duplexes binding, there is no indication for sequence-specific features recognized by dsRBDs. It appears that structural features of RNA duplexes make them appropriate RNase III substrates. Proteins which contain more than one dsRBD (for example Staufen, with 5 dsRBDs), may possibly exploit the differences between the individual dsRBDs to provide a greater potential for substrate discrimination. The observed great specificity for dsRNA cleavage by bacterial RNAse III comes from the presence or absence of anti-determinants and most likely is achieved by specific interaction of the catalytic core and not the dsRBD of the enzyme with the substrate. The presence

of only one dsRBD in class II and class III RNase III enzymes (although two identical dsRBDs are present in a homodimeric bacterial RNase III) may limit possibilities to distinguish between different substrates. Possibly the dsRBD protein cofactors of eukaryotic Drosha and Dicer enzymes provide additional specificity in substrate recognition.

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CHAPTER 1

Single Processing Center Models for Human Dicer and Bacterial RNase III

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Single Processing Center Models for Human Dicer and Bacterial RNase III

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Summary

Dicer is a multidomain ribonuclease that processes double-stranded RNAs (dsRNAs) to 21 nt small interfering RNAs (siRNAs) during RNA interference, and excises microRNAs from precursor hairpins. Dicer contains two domains related to the bacterial dsRNA-specific endonuclease, RNase III, which is known to function as a homodimer. Based on an X-ray structure of the *Aquifex aeolicus* RNase III, models of the enzyme interaction with dsRNA, and its cleavage at two composite catalytic centers, have been proposed. We have generated mutations in human Dicer and *Escherichia coli* RNase III residues implicated in the catalysis, and studied their effect on RNA processing. Our results indicate that both enzymes have only one processing center, containing two RNA cleavage sites and generating products with 2 nt 3′ overhangs. Based on these and other data, we propose that Dicer functions through *intra*molecular dimerization of its two RNase III domains, assisted by the flanking RNA binding domains, PAZ and dsRBD.

Introduction

Dicer is a large endoribonuclease responsible for processing double-stranded RNAs (dsRNAs) to ~20 bp-long small interfering RNAs (siRNAs) acting as effectors during RNA interference (RNAi), and also for excision of microRNAs (miRNAs) from the hairpin precursors. Being a key enzyme for RNAi and for miRNA function, Dicer proteins have been found in all eukaryotes studied to date, with the exception of baker's yeast. The number of genes encoding Dicer-like proteins varies from four in *Arabidopsis* to one in vertebrates (reviewed by Hannon and Zamore, 2003). Mutations in Dicer proteins in different organisms have developmental phenotypes (reviewed by Hannon and Zamore, 2003; Schauer et al., 2002), likely resulting from the compromised formation of miRNAs (Carrington and Ambros, 2003). In zebrafish and mouse, the Dicer-encoding gene is essential (Bernstein et al., 2003; Wienholds et al., 2003).

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Dicers are approximately 200 kDa multidomain proteins. Typically, their domains include a DExH RNA helicase/ATPase domain, the DUF283 and PAZ signatures, two neighboring RNase III-like domains (RIIIa and RIIIb), and a dsRNA binding domain (dsRBD) (Figure 1A). Although no mutagenesis has been performed to date on Dicer proteins, the dsRBD and RNase III domains are most certainly involved in dsRNA binding and cleavage. This is supported by the findings that ~20 bp products of Dicer processing contain 2 nt 3′ overhangs and 5′-p and 3′-OH termini, characteristic features of RNase III-mediated reactions (Elbashir et al., 2001; Nicholson, 2003). Functions of the remaining Dicer domains are not known. The PAZ domain is also found in Dicer-interacting proteins involved in RNAi and miRNA function, referred to as *P*AZ and *P*iwi *D*omain (PPD) proteins (reviewed by Carmell et al., 2002). Structural studies of the PAZ domain of the *Drosophila* PPD proteins Ago1 and Ago2 revealed similarity to the oligonucleotide binding (OB) fold, consistent with the RNA binding activity of the domain (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). The presence of the helicase/ATPase domain could be related to the findings that generation of siRNAs by *C. elegans* and *Drosophila* Dicers is greatly stimulated by the addition of ATP (Bernstein et al., 2001; Ketting et al., 2001; Liu et al., 2003; Nykänen et al., 2001). However, no such effect is observed for the mammalian enzyme (Zhang et al., 2002). Moreover, Dicer of *Dictyostelium* is devoid of the helicase/ATPase domain (Martens et al., 2002).

Irrespective of the specific role of individual domains, Dicer emerges as a very complex and dynamic enzyme, interacting with other cellular proteins. Apart from Ago proteins, shown recently to associate with Dicer directly (Tahbaz et al., 2004), these are two related small proteins, R2D2 of *Drosophila* (Liu et al., 2003) and RDE-4 of *C. elegans* (Tabara et al., 2002), and the *Drosophila* ortholog of the human fragile X mental retardation protein, dFMR1 (Ishizuka et al., 2002). Recent studies with recombinant human and *Drosophila* Dicers revealed some additional properties of the enzyme (Liu et al., 2003; Provost et al., 2002; Zhang et al., 2002). For example, the human enzyme has a strong preference for cleaving siRNAs from the ends of dsRNA substrates. In addition, activity of both the endogenous and recombinant human Dicer is strongly stimulated by proteolysis, suggesting that access to the catalytic center of the enzyme is perhaps regulated by other domains of the protein (Zhang et al., 2002).

Sequence similarity to RNase III was crucial for identification of Dicer as a protein involved in generating siRNAs (Bass, 2000; Bernstein et al., 2001; Billy et al., 2001; Ketting et al., 2001). According to current classification (Blaszczyk et al., 2001; Nicholson, 2003), Dicer belongs to the class 3 enzymes of the RNase III superfamily. The class 1 members include RNases III from bacteria and fungi, and the class 2 encompasses metazoan RNases III, exemplified by Drosha. In contrast to Dicer and metazoan RNases III, prokaryotic and lower eukaryotic RNases contain one RNase III domain (Figure

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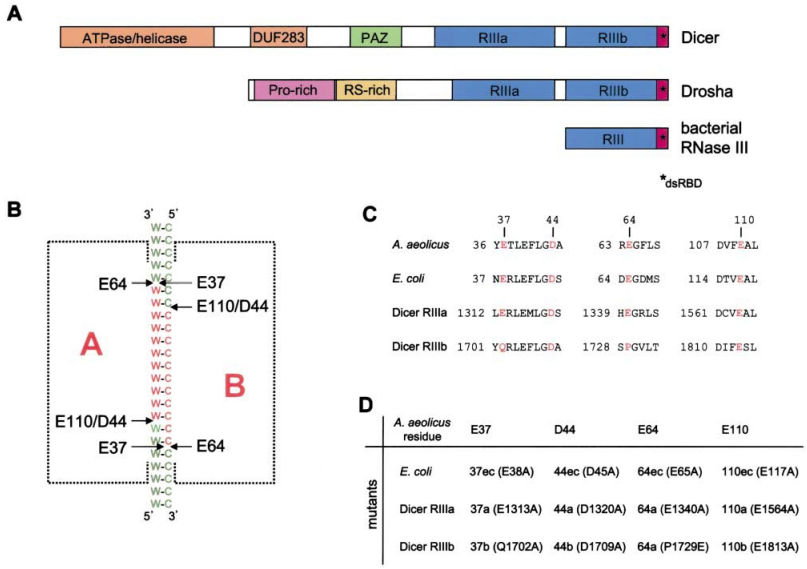

Figure 1. Schemes of the RNase III Superfamily Proteins and Summary of RNase III and Dicer RNase III Domain Sequences and Mutants

(A) Three classes of the RNase III family proteins represented by human Dicer (class 3), human Drosha (class 2), and bacterial RNase III (class 1). Individual protein domains are indicated in different colors.

(B) The postulated mechanism of dsRNA cleavage by Aa-RNase III (Blaszczyk et al., 2001). (A) and (B) represent two subunits of the homodimer enzyme, which was proposed to bind dsRNA (shown as a stack of the Watson-Crick [WC] base pairs) in the intersubunit cleft and to contain two compound catalytic centers. Residues E37 and E64, and D44 and E110 of each center, proposed to be responsible for the generation of products with 2 nt 3′ overhangs, are indicated. Residues E40 and D107, involved with D44 and E110 in coordinating the metal ion, are not shown.

(C) Alignment of conserved regions of Aa-RNase III (SwissProt accession O67082), Ec-RNase (P05797), and Dicer domains RIIIa and RIIIb (Q9UPY3; Zhang et al. 2002), containing residues equivalent to the Aa-RNase III E37 and D44, E64 and E110 (in red).

(D) Summary of single amino acid mutants in RNase III domains of human Dicer and Ec-RNase III.

1A). However, they function as homodimers, as established by biochemical work (reviewed by Nicholson, 2003) and structural studies on the bacterium *Aquifex aeolicus* (Aa) RNase III (Blaszczyk et al., 2001). Based on the X-ray structure of the catalytic domain of Aa-RNase III and mutagenesis of the *Escherichia coli* RNase III (Ec-RNase III), Blaszczyk et al. (2001) proposed a model of the dsRNA cleavage by the enzyme. In this model, shown schematically in Figure 1B, the RNase III dimer contains two compound catalytic centers, positioned at the ends of the postulated dsRNA binding cleft, each cutting two nearby phosphodiester bonds, positioned on opposite RNA strands. Within each catalytic center, two clusters of acidic residues, one comprising Glu40, Asp44, Asp 107, and Glu110, and another residues Glu37 and Glu64, would be responsible for cleavage of individual diester bonds. The former four residues coordinate single metal ions, Mn^{2+} or Mg^{2+}, present in each enzyme monomer. Based on the spacing of the catalytic centers, the model predicts generation of 9 bp products with 2 nt 3′ overhangs, consistent with the size of products generated by RNase III in vitro (Blaszczyk et al., 2001).

The bacterial RNase III structure and activity models raised many speculations regarding the mechanism of dsRNA cleavage by Dicer and the need to explain the size difference—approximately 10 versus 20 bp—in the products of RNase III and Dicer reactions. Is Dicer functioning as a pseudodimer, with RIIIa and RIIIb domains of one molecule interacting with each other, or as a true dimer in which RIII domains associate together intermolecularly in either homologous or heterologous combinations? Irrespective of the model, the size difference between RNase III and Dicer products was suggested to be due to inactivation of one of the two catalytic centers, with an evolutionarily conserved glutamate changed to proline in the position equivalent to the Aa-RNase III Glu64, a putative catalytic residue (Blaszczyk et al., 2001; Hannon, 2002; Nicholson, 2003; Zamore, 2001; see Figure 1C).

We generated mutations in all human Dicer and Ec-RNase III residues implicated in the catalysis and studied their effect on processing of dsRNA and hairpin substrates. Our results demonstrate that both enzymes have only one dsRNA processing center, containing two catalytic sites and generating products containing 3′-protruding ends. We also studied sedimentation properties of the human Dicer and the effect of mutations in its PAZ and dsRBD domains. Collectively, our data indicate that Dicer functions as an intramolecular dimer of RIIIa and RIIIb domains, assisted by two RNA

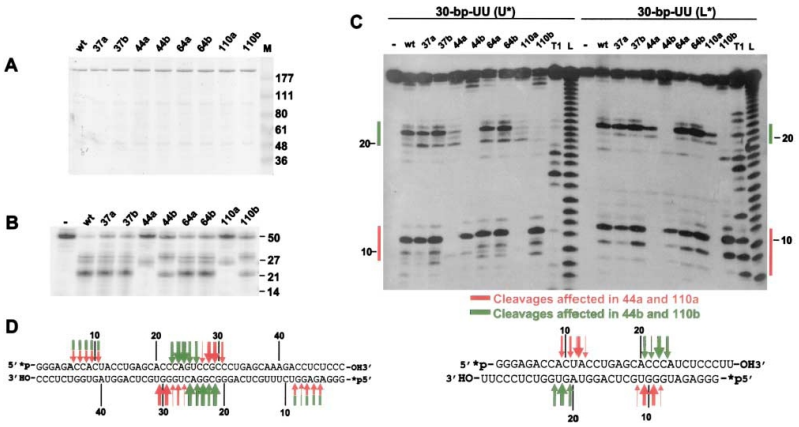

Figure 2. Activity of wt and Mutant Dicer Proteins

(A) SDS-8% PAGE of purified proteins. The gel was stained with GelCode Blue Stain (Pierce). Lane M, protein size markers (in kDa).

(B) Processing of the internally ^{32}P-labeled 50 bp dsRNA. Lane "-",dsRNA incubated without addition of the protein. Positions of oligoribonucleotide markers are indicated.

(C) Processing of the 30 bp dsRNA containing 2 nt 3′ overhangs and ^{32}P-labeled at the 5′ end of either upper (30 bp U*) or lower (30 bp L*) strands. Lanes T1 and L, RNase T1 and alkaline ladders. Positions of cleavages affected in 44a and 110a, and 44b and 110b mutants are indicated in red and green, respectively and marked in the scheme in the lower image. Thickness of arrows represents cleavage intensity.

(D) Schematic representation of processing of the 50 bp dsRNA substrate ^{32}P-labeled at the 5′ end of either the upper or lower strand. For the autoradiogram of the gel, see Supplemental Figure S3 available on *Cell* website. Cleavages marked with the composite red/green arrows represent secondary events occurring only when processing of both strands in the central region of the substrate has taken place (see text and Figure S3).

binding domains, dsRBD and PAZ, the latter domain being likely involved in the recognition of the 3′-overhang end.

Results

Processing of dsRNAs by Dicer RNase III Domain Mutants

For insight into the mechanism of Dicer cleavage we generated single amino acid mutations in its residues equivalent to the Glu37, Asp44, Glu64, and Glu110, which have been proposed to comprise the catalytic centers in Aa-RNase III. With the exception of Dicer residue Pro1731, which was substituted by glutamine, all other residues were changed to alanine. To simplify mutant description and to facilitate comparison of the Aa-RNase III, Ec-RNase III, and human Dicer data, we follow the *Aquifex* protein numbering and refer to the two RNase III domains of Dicer as RIIIa and RIIIb. Accordingly, mutations in Dicer residues Glu1313, Asp1320, Glu1340, and Glu1652 are referred to as 37a, 44a, 64a, and 110a, respectively, and mutations in residues Gln1702, Asp1709, Pro1729, and Glu1813, as 37b, 44b, 64b, and 110b, respectively (for summary of all RIII domain mutations, see Figure 1D). Mutant proteins were overexpressed in insect cells and purified as described previously (Zhang et al., 2002), yielding preparations of comparable purity (Figure 2A).

Activity of wild-type (wt) and mutant proteins was first tested with the 50 bp and 70 bp internally ^{32}P-labeled dsRNA substrates, which were previously shown to undergo effective processing by the recombinant human enzyme. Surprisingly, four of the mutant proteins (37a, 64a, 37b, and 64b) processed the substrates into 21 nt fragments with an activity comparable to that of the wt Dicer (Figure 2B, Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/118/1/57/DC1, and data not shown). The remaining mutants were considerably less active. With the 50 bp substrate, which gives a clearer readout, mutants 44b and 110b yielded lower levels of ~21 nt RNAs and also products of ~29 nt, while mutants 44a and 110a only generated products having length of 25–27 nt. Notably, each pair of mutants appeared to generate a subset of products formed by the wt enzyme (Figure 2B, and see below).

We also generated three sets of double mutants in Dicer RIII domains. Mutants 44a110a and 44b110b combine mutations 44 and 110 in Dicer domains RIIIa and RIIIb, respectively. Mutants 44ab and 110ab combine mutations in equivalent positions in each Dicer RIII domain, and mutants 37ab and 64a37b are combinations of mutations which had no apparent effect on Dicer activity. 44ab110ab is a quadruple mutant combining mutations 44a110a and 44b110b. Mutants 44ab and 110ab, and the quadruple mutant were found to be completely inactive, while other double mutants mimicked precisely the activity of the single amino acid mutants from which they were derived; identical results were obtained for both the 70 bp (Supplemental Figure S1 available on *Cell* website) and the 50 bp (data not shown) substrates.

We have demonstrated previously that Dicer preferen-

tially cleaves off siRNAs from the termini of dsRNA substrates (Zhang et al., 2002). We took advantage of this observation to map cleavage sites introduced by the wt and mutant proteins in the 30 bp substrate. The 30 bp dsRNA can be cleaved by Dicer only once yielding the ~21 nt siRNA-like products and the cut-off fragments (Zhang et al., 2002; and our unpublished results). The 30 bp RNA can be accessed by Dicer from either end but the resulting "end-specific" cleavages can be monitored independently from each other when single strands of the substrate are labeled at the terminus. As shown in Figure 2C, incubation of the wt Dicer with the 30 bp substrate containing either the upper or lower strand labeled at the 5′ terminus yielded series of processing products diagnostic of the enzyme approaching the substrate either from the label-containing (20–23 nt fragments) or opposite (9–12 nt fragments) end. Consistent with the analysis of the internally labeled substrates (see Figure 2B), processing of the 5′-end-labeled 30 bp RNA was unaffected by mutations 37a, 64a, 37b, and 64b (Figure 2C). In contrast, mutations 44a and 110a prevented processing at the labeled-end proximal but not distal sites, while the reverse was true for mutations 44b and 110b. This was irrespective of whether the 5′ label was on the upper or the lower strand of the substrate (Figure 2C). The 30 bp substrates used in this experiment contained 2 nt 3′ overhangs at both ends but similar results were obtained with the blunt-ended 30 bp dsRNA (data not shown). Likewise, processing of the terminally labeled 30 bp dsRNA substrate with a different sequence and base composition yielded a very similar cleavage pattern (Supplemental Figure S2 available on *Cell* website).

The most straightforward interpretation of the data presented above is that residues equivalent to the Aa-RNase III Asp44 and Glu110 of both Dicer RNase III domains are part of one processing center responsible for the dsRNA cleavage, with residues of the domain RIIIa being required for the cleavage of one RNA strand and residues of RIIIb for the nearby cleavage of the second strand, resulting in the formation of products with 3′ overhangs. This conclusion is further supported by analysis of terminally labeled 50 bp substrate processing by Dicer mutants (Figure 2D; for autoradiogram of the gel, see Supplemental Figure S3 available on *Cell* website). This analysis also provides an explanation for the differences in the length of processing products of the internally labeled 50 bp dsRNA generated by the RIIIa and RIIIb domain mutants (see Figure 2B). Since inactivating mutations in domain RIIIa, 44a and 110a, prevent cleavage at sites separated by ~21 nt from RNA 3′-hydroxyl ends (sites shown in red in Figure 2D), the only products formed are those resulting from processing at the nearby sites (shown in green) on complementary strands; for the substrate containing blunt ends, as with the 50 bp dsRNA, these products are expected to be approximately 23 to 27 nt in length, consistent with the data shown in Figure 2B. Conversely, the inactivating mutations in the domain RIIIb, 44b and 110b, prevent cleavage at sites close to the center of the dsRNA (sites shown in green in Figure 2D), and only the products of processing at sites on the complementary RNA strands (shown in red in Figure 2D) accumulate; these products are expected to have lengths of approximately 21 and 29 nt, also in agreement with the data in Figure 2B.

Analysis of terminally labeled 50 bp substrates revealed additional sets of cleavages, marked with the composite red/green arrows in Figure 2D. Positions of these cleavages and the fact that they are observed with the wt protein and its mutants 37 and 64, but not mutants 44 and 110, in both RIII domains (see Supplemental Figure S3 available on *Cell* website), indicate that they represent secondary events occurring only when processing of both strands in the central region of the substrate has taken place. The requirement for the primary processing event explains why no labeled products diagnostic of the cleavage at complementary sites on opposite RNA strands could be identified (Supplemental Figure S3 available on *Cell* website).

Processing of Pre-*let-7* RNA by Dicer Mutants

We extended the analysis of Dicer RIII domain mutants to miRNA precursors, which represent another class of cellular substrates of Dicer (Hannon and Zamore, 2003). Long primary transcripts containing miRNA sequences are first processed in the nucleus by Drosha into ~70 nt long hairpins referred to as pre-miRNAs. The pre-miRNAs are then matured by Dicer to ~21 nt miRNAs in the cytoplasm (Lee et al., 2003). We generated the precursor of *let-7* miRNA, expected to correspond to the Drosha cleavage product (Basyuk et al., 2003; Lee et al., 2003; see Figure 3C), by the self-processing of the in vitro hybrid transcript, which contains the hammerhead ribozyme upstream of the pre-*let-7* sequence. Processing of the internally labeled pre-*let-7* RNA by the wt protein yielded the double-stranded siRNA-like product, as established by gel electrophoresis under nondenaturing conditions (Figure 3A). Analysis of processing of pre-*let-7* RNA, terminally labeled at either the 5′ or 3′ end, by different Dicer mutants revealed that the cleavage is unaffected by mutations 37a, 64a, 37b, and 64b, and that mutations 44a and 110a, and 44b and 110b strongly inhibit processing at adjacent sites on the descending and ascending hairpin arms, respectively (Figures 3B and 3C). These data corroborate conclusions derived from the dsRNA substrate experiments. Collectively, they demonstrate that residues equivalent to the Aa-RNase III Asp44 and Glu110 of both Dicer RIII domains are part of one processing center containing two RNA cleavage sites functioning independently of each other, and that amino acids equivalent to Aa-RNase III residues Glu37 and Glu64 are not essential for the catalysis. The data also indicate that during both the pre-*let-7* RNA (Figure 4) and dsRNA (e.g., see Figure 2D) processing, Dicer accesses the substrate in a polar fashion, with the RIIIa domain always cutting the RNA strand bearing a 3′-hydroxyl, at approximately 21 nt from the end.

Activity of the *E. coli* RNase III Mutants

The Dicer mutagenesis and dsRNA and pre-miRNA processing data are inconsistent with the proposed model of dsRNA cleavage by the bacterial RNase III. They are also in conflict with some previous experimental data indicating that the Ec-RNase III residues equivalent to

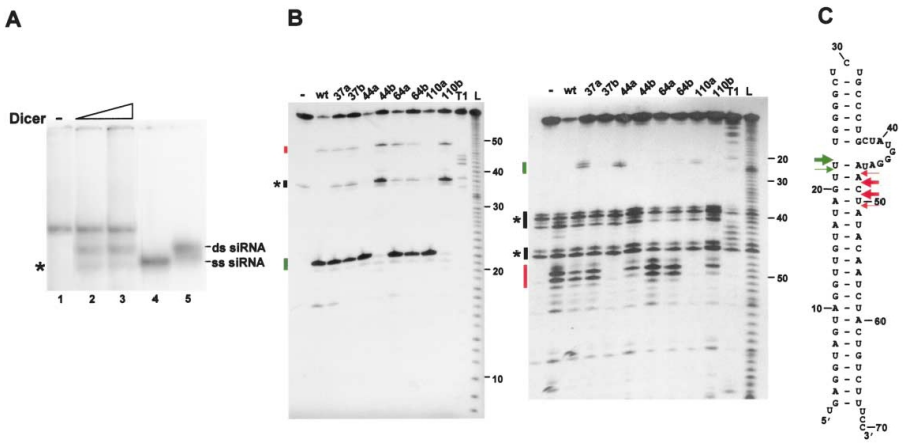

Figure 3. Processing of Pre-*let*-7 RNA by Recombinant Dicer Enzymes

(A) Processing of internally ^{32}P-labeled pre-*let*-7 RNA by the wt Dicer monitored by nondenaturing PAGE. The substrate (3 fmol) was incubated in the absence (lane 1) or presence (lanes 2 and 3, containing 10 and 30 ng of Dicer, respectively). Lanes 4 and 5, 21 nt single-stranded and double-stranded siRNA markers, respectively. The band marked with the asterisk corresponds the top region of the hairpin.

(B) Mapping of cleavage sites in the 5′-end (left image) or 3′-end (right image) ^{32}P-labeled pre-*let*-7 RNA. The RNA was incubated in the absence (lanes "-") or presence of indicated Dicer preparations. Dicer-induced cleavage sites on ascending and descending hairpin arms are indicated by green and red bars, respectively. Unspecific, nonenzymatic cleavages at UA and UG phosphodiesters in the bulge are marked with asterisks.

(C) Secondary structure of pre-*let*-7 RNA and positions of cleavage sites as determined in B. Cleavages affected by mutations 44a and 110a, and 44b and 110b, are in red and green, respectively.

the Aa-RNase III Glu37 and Glu64 are essential for the catalysis. Since the latter conclusion was based on a rather indirect assessment of effects of mutations on enzyme activity, namely determining the Ec-RNase III-dependent expression of the λ*N-lacZ* reporter in vivo (Blaszczyk et al., 2001), we reinvestigated activity of Ec-RNase III mutants by more direct methods. Four single amino acid mutants of Ec-RNase III (Glu38Ala, Asp45Ala, Glu65Ala, and Glu117Ala, referred to as 37ec, 44ec, 64ec, and 110ec, respectively; see Figure 1D), in positions corresponding to Aa-RNase III residues Glu37, Asp44, Glu64, and Glu110, were constructed and proteins overexpressed and purified (Figure 4A). Their activity was compared with that of the wt protein, using dsRNA, R1.1 RNA, and R1.1[WC] RNA as substrates. R1.1 and R1.1[WC] RNAs, shown in Figure 4E, represent well-characterized Ec-RNase III substrates. R1.1 RNA corresponds to the phage T7 RNA fragment processed only at the descending arm of the hairpin, and R1.1[WC] RNA is modified such that it is processed at both hairpin arms (Li and Nicholson, 1996; Nicholson, 2003).

Reactions with the internally labeled 70 bp (Figure 4B) and 50 bp (data not shown) dsRNAs revealed that mutations 44ec and 110ec very strongly compromise activity of the enzyme; low levels of products and intermediates were only observed when a large excess of mutant proteins was used. This is consistent with the previous findings that mutation of Glu117 (residue substituted in the 110ec mutant) strongly inhibits activity of the *E. coli* enzyme (Nicholson, 2003; and references therein). In contrast, mutants 37ec and 64ec showed activity comparable with that of the wt protein (Figure 4B).

Terminally labeled 30 bp dsRNA, and R1.1 and R1.1[WC] RNAs were used to map cleavage sites by wt and mutant *E. coli* proteins (Figures 4C–4E). The wt Ec-RNase III cleaved 30 bp dsRNA in the central region and, as expected, processed R1.1 and R1.1[WC] RNAs at the descending arm and at both the ascending and descending arms, respectively. Mutants 37ec and 64ec cleaved all substrates in a manner similar to the wt protein, while mutants 44ec and 110ec were inactive. These findings are consistent with the results of Dicer mutagenesis and indicate that the bacterial RNase III, which functions as a homodimer, also has only one processing center, with the Ec-RNase III residues Asp45 and Glu117 (equivalent to Aa-RNase III residues Asp44 and Glu110) of each monomer contributing to the cleavage of two diester bonds separated by two base pairs and present on opposite RNA strands.

We used the R1.1 single cleavage substrate to compare kinetic parameters of the wt Ec-RNase III with that of its mutants 37ec and 64ec. V_{max} values for all three proteins were similar but K_m values for mutants 37ec and 64ec were, respectively, 5 and 3 times higher than the 137 nM value determined for the wt enzyme (Supplemental Table S1 available on *Cell* website), suggesting a small deficiency in the substrate binding. It is possible that this deficit of mutant proteins is an underlying factor explaining the inability of similar mutants to process the λ*N-lacZ* reporter in vivo as reported by Blaszczyk et al. (2001). (We note that in this work only one of the Glu residues was mutated to alanine; the other was mutated to valine.) Since the λ*N-lacZ* reporter processing was measured following the shift of bacteria to 42°C (Blasz-

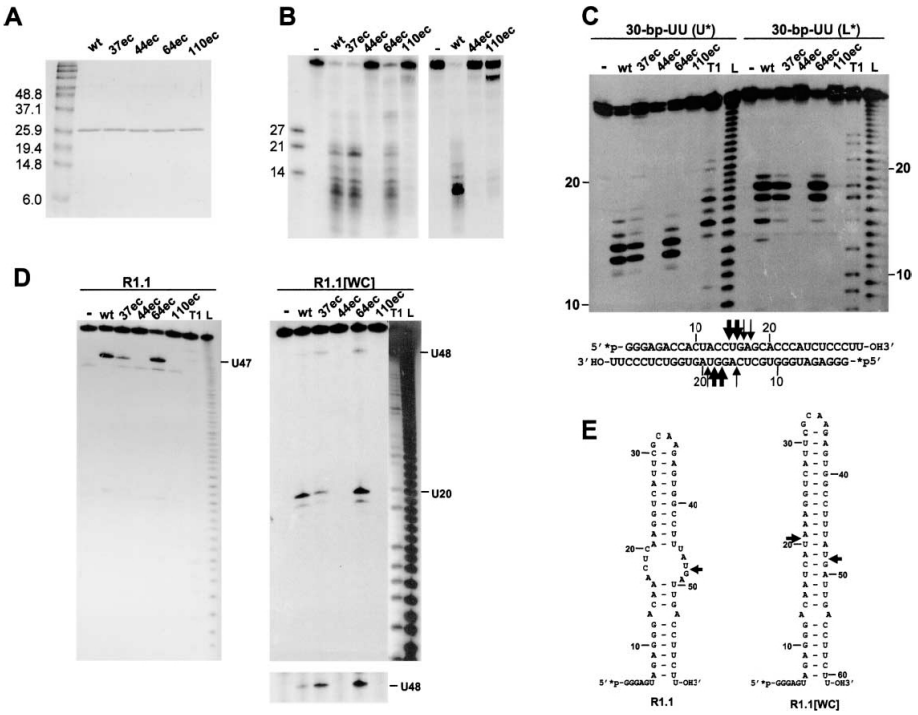

Figure 4. Activity of Recombinant wt and Mutant Ec-RNases III

(A) The purified wt or mutant proteins (0.5 μg) were analyzed by SDS-15% PAGE. Lane M, size markers.

(B) Processing of the 70 bp internally labeled dsRNA (3 fmol). Assays contained either 10 ng (left image) or 70 ng (right image) of the indicated protein.

(C) Processing of the 30 bp dsRNA containing 2 nt 3′ overhangs and terminally labeled on either the upper (30 bp U*) or lower (30 bp L*) strand. Cleavage positions are shown in the bottom image.

(D) Processing of the 5′-^{32}P-labeled R1.1 (left image) and R1.1[WC] (right image) RNAs. RNase T1 and alkaline ladders in the right image represent longer exposure of the same gel. Cleavage positions at U_{47} (R1.1) and U_{20} and U_{48} (R1.1[WC]) are indicated; assignment of cleavages to U_{47} and U_{48} was confirmed by long migration gels. Longer exposure of the U_{48} region is shown at the bottom.

(E) Structure of R1.1 and R1.1[WC] RNAs with marked cleavage sites.

czyk et al., 2001), we have tested whether mutant proteins retain activity at this temperature. The 37ec and 64ec mutants processed dsRNA at 37°C and 42°C with comparable activity (data not shown).

Gradient Sedimentation Analysis of Dicer

As shown above, each of the two RNase III domains of Dicer, RIIIa and RIIIb, contributes amino acids to one processing center generating products with a short 3′ overhang. Thus, Dicer likely operates as a monomer, which functionally represents an intramolecular pseudodimer. We used glycerol gradients to determine whether Dicer sediments as a monomer or a larger complex. Analysis of purified Dicer indicated that the enzyme fractionates at the position corresponding to a molecular mass of ~180 kDa (Figure 5A). The enzyme in a complex with the 30 bp dsRNA substrate having 3′ overhangs sedimented at similar position (Supplemental Figure S4 available on *Cell* website), as did Dicer present in the extract prepared from insect cells overproducing the protein (data not shown). We also analyzed properties of endogenous Dicer in extracts of mouse teratocarcinoma P19 cells (Billy et al., 2001). The protein was found to sediment at ~230 kDa, suggesting that it may be associated with other cellular component(s) of a relatively small size (Figure 5B). Shorter centrifugation of the gradient revealed that an appreciable amount of Dicer also sediments in a region of 80S or larger, suggesting that a fraction of Dicer in P19 cells is associated with ribosomes or another large complex (Figure 5C). The data support a model in which domains RIIIa and RIIIb of a single Dicer molecule interact together to form an intramolecular pseudodimer.

Activity of Mutants in Dicer PAZ and dsRBD Domains

Human Dicer and most of its orthologs in other species contain a PAZ domain positioned N-terminally to RIIIa (Hannon and Zamore, 2003; Schauer et al., 2002). This approximately 150 amino acid long domain is also found in PPD proteins (Carmell et al., 2002), and structural studies of the PAZ domain of *Drosophila* PPD proteins

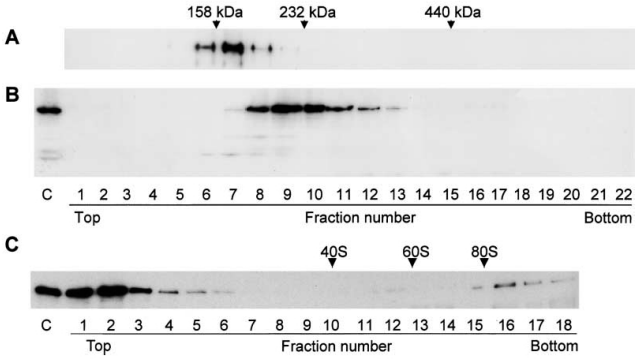

Figure 5. Sedimentation of Purified Dicer (A) and P19 Cell Extract (B and C) on 10%–30% Glycerol Gradients

Position of protein markers (aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa), and ribosomal subunit and 80S ribosome markers, run in parallel gradients, are indicated. Lanes C, fraction of the input extract. Dicer was visualized by Western blotting. Centrifugation was for 20 hr in (A) and (B), and for 4.5 hr in (C).

Ago1 and Ago2 revealed similarity to the OB fold, consistent with the RNA binding activity of the domain (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). Three Dicer mutant proteins were overexpressed and purified to test the importance of the PAZ domain for RNA processing. In mutants F960A and YY971/2AA, conserved aromatic amino acids, Phe960, and Tyr971 and Tyr972, respectively, are replaced by alanine. Equivalents of the mutated residues were demonstrated to be important for optimal RNA binding of the Ago protein PAZ domains (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003) (for an alignment of PAZ domains of Dicer and PPD proteins, see Supplemental Figure S5 available on *Cell* website). The third mutant is E1036A. Equivalent mutation in *Drosophila* Ago1 had no effect on RNA binding activity (Yan et al., 2003), but in Ago2, the mutation of the corresponding Glu residue to Lys rendered the PAZ protein insoluble (Lingel et al., 2003). In addition, Dicer protein with the C-terminal dsRBD domain deleted, ΔdsRBD, was prepared. When assayed with the 70 bp blunt-ended dsRNA as a substrate, all four mutations markedly decreased Dicer activity. Mutations of the two adjacent tyrosines in the PAZ domain and deletion of the dsRBD had the strongest effect, lowering activity of the enzyme approximately 10-fold (Supplemental Figure S6 available on *Cell* website).

Binding studies with the PAZ domain of Ago proteins indicated that it may recognize the 3′-protruding ends of siRNAs (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). Thus, we compared activity of the wt and mutant Dicer proteins in processing of 30 bp substrates containing either blunt (30 bp) or 2-nt 3′-overhang (30 bp-UU) ends. The wt protein processed the 30 bp-UU RNA with nearly 2-fold higher efficiency than the 30 bp RNA, and each of the three PAZ mutations had a 1.4- to 1.9-fold stronger effect on processing of the 30 bp-UU than on the 30 bp substrate (Figure 6A; for quantification, see the legend). A stronger discrimination between 30 bp and 30 bp-UU substrates was displayed by the ΔdsRBD mutant. While this mutation decreased the cleavage of the 30 bp-UU RNA only 1.9-fold, it decreased 4-fold the cleavage of the 30 bp RNA (Figure 6B). We also compared ability of the wt Dicer and the ΔdsRBD mutant to process the *let-7* RNA precursor containing either 2 nt 3′-protruding (pre-*let-7*) or blunt (pre-*let-7*$_{BL}$) ends. As with the dsRNA substrates, the wt protein showed an ~2.5-fold preference for the pre-*let-7* RNA with the 3′ overhang, and the processing of this substrate was less strongly affected by the dsRBD deletion than the processing of pre-*let-7*$_{BL}$ (Figure 6C). Of note, we found that processing of pre-let-7 RNA by Dicer is not affected by the absence of a phosphate on the recessed 5′-end (data not shown). Taken together, these results indicate that Dicer has a preference for processing of substrates bearing 3′ overhangs; this preference gets accentuated by the deletion of dsRBD. Moreover, the results support a role for the Dicer PAZ domain in the recognition of the substrate 3′-protruding end (see Discussion).

Discussion

Our data indicate that both the human Dicer and the bacterial RNase III contain only a single dsRNA processing center responsible for cleavage of two nearby phosphodiester bonds on opposite RNA strands, generating products containing 2 nt 3′-protruding ends. Residues equivalent to the Aa-RNase III Asp44 and Glu110 of two RNase III domains contribute to this active center, while residues equivalent to Glu37 and Glu64, previously proposed to be required for the RNA cleavage (Blaszczyk et al., 2001), were found to be nonessential. Dispensability of Glu37 and Glu64 equivalents for the catalysis is further supported by the recent finding that mutation of these two residues in the RIIIa domain of the *Drosophila* Dicer does not inactivate the enzyme (Lee et al., 2004). Since in the case of Dicer, two different RNase III domains, RIIIa and RIIIb, contribute to the processing center, analysis of Dicer proteins bearing mutations in individual RIII domains was particularly revealing. This analysis, performed with both dsRNA and pre-*let-7* substrates, demonstrated that mutations of Asp44 and Glu110 equivalents in the RIIIa domain prevent cleavage of one RNA strand but have no effect on cutting the other, while the reverse is true for mutations in the RIIIb domain. Hence, the two catalytic sites present in the RIIIa/RIIIb dimer function independently of each other.

Simultaneous mutations in both Dicer RIII domains (mutants 44a44b or 110a110b) abolished cleavage of each RNA strand. These Dicer double mutants are functionally equivalent to the Ec-RNase III single amino acid

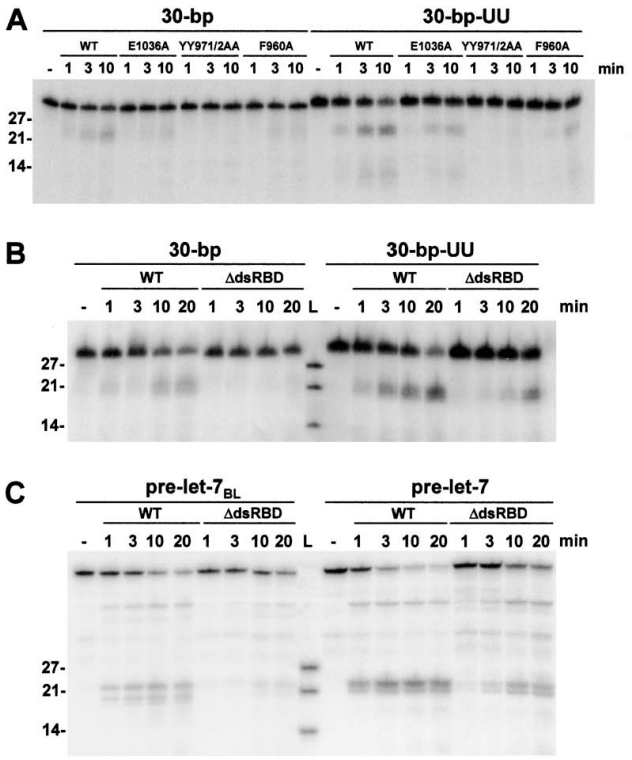

Figure 6. Activity of Dicer PAZ and dsRBD Domain Mutants

(A) Kinetics of processing of the internally labeled 30 bp dsRNA containing either blunt ends (30 bp) or 2 nt 3′ overhangs (30 bp-UU) by the wt Dicer and the PAZ domain mutants. Proteins added to the reaction and times of incubation are indicated at the top. PhosphorImager quantification revealed that the wt Dicer cleaves 1.92-fold and 1.54-fold more of the input 30 bp-UU then 30 bp RNA at time points 3 min and 10 min, respectively (ratios based on averages of three independent experiments similar to those shown in (A) and (B). The PAZ mutations E1036A, YY971/2AA, F960A had 1.4-, 1.4-, and 1.9-fold stronger effect on processing of 30 bp-UU then 30 bp RNA, respectively (values represent averages of 3 and 10 min time points from two independent experiments). Note that the 30 bp-UU RNA contains four additional U residues (see Figure 2C), which are labeled with ^{32}P. Appropriate corrections were made to calculate moles the substrate and the reaction products. (B and C) Comparison of activities of wt and ΔdsRBD Dicers to cleave 30 bp and 30 bp-UU substrates (B) and pre-*let-7* RNA containing either blunt (pre-*let-7*$_{BL}$) or 3′-protruding (pre-*let-7*) ends (C). In (B), the ΔdsRBD mutation decreased cleavage of the 30 bp-UU RNA 1.9-fold, and cleavage of 30 bp RNA 4.0-fold (averages from three independent experiments). Reactions in (A–C) contained 40 ng of Dicer proteins and 3 fmol of RNA.

mutants 44ec and 110ec. Since bacterial RNase III functions as a homodimer (Nicholson, 2003), the mutations affect processing of both RNA strands. Notably, mutation 44ec inhibited RNA cleavage more severely than mutation 110ec. It is possible that Asp44 (Asp45 in Ec-RNase III; changed to alanine in the 44ec mutant), which is engaged in metal ion binding via the water molecule in Aa-RNase III (Blaszczyk et al., 2001), acts as a general base that deprotonates the metal bound water (see also Nicholson, 2003).

Based on the results of RNase III and Dicer mutagenesis, and other findings presented in this work, we propose new models for substrate cleavage by both enzymes. In the bacterial Aa-RNase III model (Figures 7A–7C, and Supplemental Figure S7 available on *Cell* website), the dsRNA substrate is rotated by approximately 30° with regard to its position in the model of Blaszczyk et al. (2001). The following arguments support the new placement of the substrate and its availability for the cleavage by the enzyme. (1) In the new position, each of the two scissile phosphodiester bonds is in the proximity of four acidic residues (Glu40, Asp44, Asp107, and Glu110), which coordinate the metal ion (Mn^{2+} or Mg^{2+}) and constitute the catalytic site for the cleavage of one strand. (2) In the model, each catalytic site of the dimer contains a metal ion required for the catalysis. The distance to the scissile bonds (see legend to Figure 7) is consistent with both metal ions participating in the catalysis, each involved in cleaving one of the two RNA strands. In the reported crystal structure of Aa-RNase III, the two metal ions coordinated by four acidic residues in each monomer are the only metal ions identified. No metal ions were found coordinated by residues Glu37 and Glu64, generating speculations about possible repositioning of the substrate in order for the second diester bond cleavage to take place (Nicholson, 2003). (3) The enzyme surface along the new dsRNA placement contains many conserved amino acid residues, likely contributing to substrate binding (Figure 7C; for positions of conserved residues in the alignment of RNase III domains of different proteins, see Supplemental Figure S8 available on *Cell* website).

In the new model, no catalytic role is assigned to residues equivalent to Aa-RNase III Glu37 and Glu64. However, these amino acids are not far from the RNA helix and could, in the presented model, contact the RNA substrate either directly or indirectly via an ion. Such a proximity might explain an increase of K_m values for Ec-RNase III mutants 37ec and 64ec. Notably, Glu37 and Glu64 residues are not 100% conserved in RNase III domains, in contrast to residues equivalent to Asp44 and Glu110. In different members of the RNase III superfamily, Glu, Gln, and Asp residues are present at the Glu37 position, and Glu, Gln, Pro, and Val residues are found at the Glu64 position. For example, in the *Thermotoga maritima* RNase III, the crystal structure of

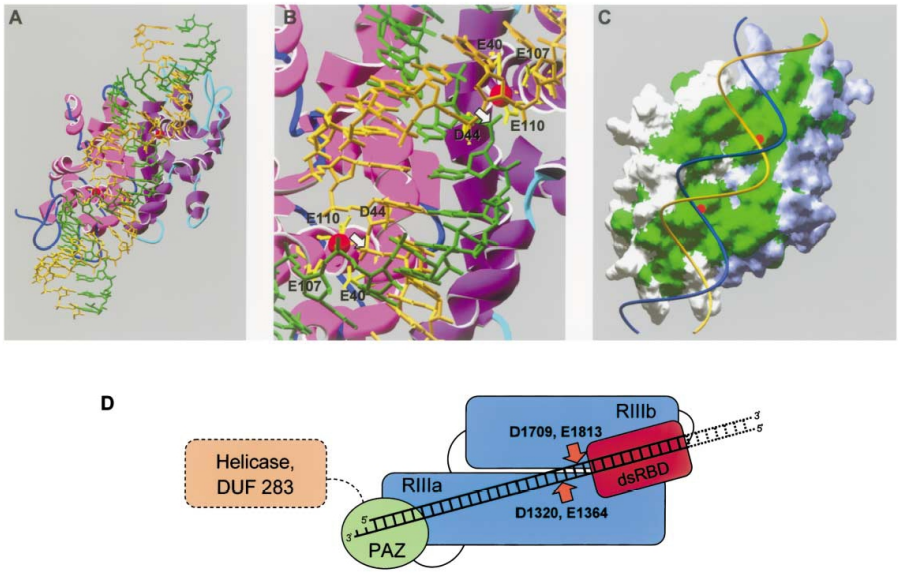

Figure 7. Models of RNase III and Dicer Interactions with dsRNA

(A and B) Frontal view of a complete (A) and an enlarged central region (B) of the Aa-RNase III catalytic domain dimer (Blaszczyk et al., 2001), interacting with the dsRNA helix representing the 30 bp dsRNA shown in Figure 2C. Proteins are represented as ribbons; the two monomers are in different shades of purple. Mn^{2+} ions are as red spheres. Residues coordinating the Mn^{2+} ion in each monomer are shown as yellow sticks. Mn^{2+} ions present in each proposed catalytic site are 21.5 Å apart, while the distance between the two scissile phosphodiester bonds (marked with arrows), across the minor groove, is 17.2 Å; Mn^{2+} ions and phosphorus atoms of cleavable bonds in the model are approximately 5 Å apart. These distances are likely to change upon dsRNA binding to the enzyme. In the same vein, we cannot exclude that additional ions are present in the activated complex. In our model, the dsRNA substrate is rotated by approximately 30° clockwise as compared to its position, parallel to the subunit interface, in the model of (Blaszczyk et al., 2001).

(C) Distribution of conserved amino acids in 75 bacterial and fungal RNase III proteins. Residues conserved in more than 70% of proteins are in green. The modeled-in dsRNA is shown as ribbons. Two monomers are shown in different colors.

(D) A model of dsRNA processing by Dicer. Individual domains of Dicer are in different colors. Helicase/ATPase and DUF283 domains, with no assigned function, are delineated with a broken line. The RIIIa domain is drawn as a larger rectangle than RIIIb to illustrate its larger size. Its placement also illustrates the fact that this domain cleaves the 3′-OH-bearing RNA strand.

which has been recently determined (Protein Data Bank ID 1O0W), Glu64 is replaced by valine. Importantly, a model of the dsRNA-protein complex similar to that for the Aa-RNase III could also be built for the *T. maritima* enzyme (our unpublished results).

Mutagenesis has demonstrated that Dicer domains RIIIa and RIIIb contribute together to one "dimeric" catalytic center, analogous to that of the bacterial RNase III homodimer. Since during gradient sedimentation, Dicer behaves as a monomer rather than a dimer, also when associated with the substrate, we propose that the two interacting RIII domains originate from a single Dicer molecule. Bacterial and fungal RNases III assemble into dimers in the absence of substrate and X-ray analysis has revealed the important contribution of hydrophobic interactions to this process (Blaszczyk et al., 2001). Sequence alignments and modeling of the conserved core regions of Dicer RIIIa and RIIIb domains indicated that interactions similar to the hydrophobic "ball-socket" contacts identified in bacterial enzymes (Blaszczyk et al., 2001; Protein Data Bank ID 1O0W) may also occur between Dicer RIIIa and RIIIb domains (our unpublished observations). Additional support for the intramolecular dimer, containing a single processing center, is offered by the observation that long dsRNA substrates are always shortened successively by ~20 bp siRNA-like units and never by longer segments of dsRNA (for example, see Figure 2 in Zhang et al., 2002). The dependence of secondary processing events on prior cleavages in the center of the 50 bp substrate (Figure 2D and Supplemental Figure S3 available on *Cell* website), also argues for the stepwise excision of the ~20 bp segments by an enzyme with a single processing center.

In most Dicer proteins, the two RIII domains are flanked by the PAZ and dsRBD domains, positioned N- and C-terminally, respectively (see Figure 1A). The PAZ domains of Dicer and PPD proteins were originally thought to mediate interaction between these proteins, but recent work has shown that RIII and Piwi domains of Dicer and PPD proteins are responsible for the interaction (Tahbaz et al., 2004). Recent structural studies of the PAZ domain of *Drosophila* PPD proteins Ago1 and Ago2 revealed its RNA binding potential (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003), raising a possibility that the PAZ domain of Dicer contributes to substrate recognition (Song et al., 2003). Indeed, we

found that mutations of four conserved amino acids, either singly (mutants F960A and E1036A) or in combination (mutant YY971/2AA), have marked inhibitory effect on the activity of the protein. A role for the PAZ domain in dsRNA processing is further supported by the finding that the purified C-terminal fragment of Dicer, encompassing RIIIa, RIIIb, and dsRBD domains, shows no dsRNA cleavage activity (our unpublished results).

We have shown previously that human Dicer preferably cleaves off siRNA products from dsRNA ends and that blocking the ends with RNA tetraloops or RNA-DNA duplexes significantly delays the cleavage reaction (Zhang et al., 2002). Moreover, studies of miRNA precursor processing in vertebrate cells have revealed that Dicer preferentially excises miRNAs from pre-miRNA hairpin intermediates produced by Drosha, possibly due to the availability of proper free ends generated by this enzyme (Lee et al., 2003). The results presented in this work directly demonstrate that Dicer not only requires free dsRNA ends but that it also cleaves substrates with 2 nt 3′-overhangs more effectively than those containing blunt ends. Although for the wt Dicer this preference was found to be relatively small, deletion of the dsRBD domain made the enzyme more dependent on the presence of the 3′-overhang during the processing of both dsRNA and pre-*let-7* RNA substrates. Most probably, the deletion of dsRBD, a domain generally known to mediate unspecific interactions with dsRNA, makes Dicer's interaction with the substrate more dependent on its specific structural features and on protein domains other than its dsRBD. Our findings that mutations in the PAZ domain strongly inhibit Dicer activity, and that this inhibition is greater for the dsRNA containing 3′ overhangs than that with blunt ends, strongly suggest that the PAZ domain of Dicer participates in the recognition of the terminal 3′ overhangs. This conclusion is in agreement with the demonstrated preference of the PAZ domain of *Drosophila* Ago1 and Ago2 for single-stranded RNAs or double-stranded siRNAs with 2 nt 3′ overhangs, as opposed to blunt-ended siRNAs (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). It is likely that the recognition of staggered RNA ends is a major physiological function of the PAZ domain in both Dicer and PPD proteins.

One of the features distinguishing reactions catalyzed by Dicer and the bacterial RNA III is the length of the products, approximately 20 bp and 10 bp, respectively. In previous models, based on the data of Blaszczyk et al. (2001), this size difference was suggested to be due to inactivation of one of the two processing centers, postulated for the RNase III class enzymes by these authors (see Introduction). Our demonstration that both Dicer and bacterial RNase III contain only one active processing center makes this explanation unlikely. Which Dicer domain(s) could be responsible for measuring the distance between the end and the cleavage site? Since mutations in the Dicer PAZ domain were found to have an effect on the processing efficiency but not on the length of the products (Figure 6A, Supplemental Figure S6 available on *Cell* website), we consider it improbable that this domain is a major size-determining factor. It is more likely that the atypical N-proximal RIII domain of Dicer, RIIIa, perhaps assisted by PAZ, measures the distance between the substrate terminus and the cleavage site. RIIIa of all Dicer proteins is much longer than the RIIIb domain and all other typical RNase III catalytic domains, including the N-proximal RNase III domain of Drosha proteins (see Figure 1A).

A model of the dsRNA cleavage by Dicer, incorporating the results of mutagenesis and gradient analyses, and all considerations discussed above, is presented in Figure 7D. The most important feature of the model is the presence of a single dsRNA cleavage center, which is formed by the RIIIa and RIIIb domains of the same Dicer molecule, and which processes the dsRNA approximately 20 bp from its terminus. The PAZ domain recognizes the substrate terminus with the 3′ overhang, and the RIIIa domain, possibly in conjunction with PAZ, measures the distance to the cleavage site. The placement of the RIII domains illustrates the asymmetry of the catalytic region, with RIIIa cleaving the 3′-hydroxyl- and RIIIb cleaving the 5′-phosphate-bearing RNA strand.

The recombinant Dicer utilized in this study is catalytically inefficient. When assayed at excessive (30–100 nM) substrate concentrations, it generates 0.5–1.0 mole of the siRNA product per mole of enzyme in a 30 min incubation (Zhang et al., 2002; our unpublished results). The low activity of purified Dicer is at least partially due to the reaction product, which remains associated with the enzyme (Zhang et al., 2002). Recent studies of the RNA-induced silencing complex (RISC) in *Drosophila* demonstrated that Dicer accompanies siRNA along the RISC assembly pathway and is an essential component of mRNA-cleaving "holo-RISC", sedimenting at ~80S (Lee et al., 2004; Pham et al., 2004; Tomari et al., 2004). The ~80S Dicer-containing complex identified in this study (Figure 5C) may represent the mammalian equivalent of *Drosophila* holo-RISC. The finding that Dicer assembles into RISC along with the siRNA offers an explanation for the low activity of the purified enzyme. In the absence of other RISC components, Dicer would remain associated with its siRNA cargo and be incapable of catalyzing multiple cleavage reactions. The finding that Dicer forms part of RISC (Lee et al., 2004; Pham et al., 2004; Tomari et al., 2004) also provides a rationale for the observation that binding of the PPD protein hAgo2 to Dicer inhibits the RNase activity of the enzyme (Tahbaz et al., 2004). Possibly, one function of Ago proteins, established components of RISC (reviewed by Hannon and Zamore, 2003), is to prevent cleavage of dsRNA by Dicer present in the complex to avoid that a single RISC is associated with more than one siRNA.

Experimental Procedures

Preparation of RNA Substrates

Unless indicated otherwise, all substrates contained 5′-p and 3′-OH ends. dsRNAs, containing either blunt or 2 nt 3′-protruding ends, were obtained by annealing of appropriate RNAs obtained by the T7 polymerase in vitro transcription. Nonradioactive RNAs were synthesized using the Ambion T7 MegaShortScript transcription kit. After transcription, samples were treated with DNase I, extracted with phenol, and purified by denaturating 10% polyacrylamide gel electrophoresis (PAGE). Following dephosphorylation by calf intestine phosphatase (CIP), RNAs were 5′-end-labeled, using T4 polynucleotide kinase and [γ-^{32}P]ATP. The internally ^{32}P-labeled dsRNAs were prepared similarly, except that transcription was performed in the presence of [α-^{32}P]UTP, and [γ-^{32}P]ATP was replaced by 10

mM cold ATP during the 5′-phosphorylation. Complementary RNA strands were annealed at 95°C for 3 min in 20 mM NaCl, transferred to 75°C, and then slowly cooled down to 20°C. The 3′-end-labeling of pre-*let-7* RNA was performed with T4 RNA ligase and [5′-^{32}P]pCp; following ligation, the 3′-phosphate was removed by treatment with CIP, and RNA purified by PAGE. Prior to use, pre-*let-7* RNA was dissolved in water and renaturated by incubation at 90°C for 1 min, followed by incubation at 25°C for 15 min in 30 mM Tris-HCl, [pH 6.8] containing 50 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, and 10% glycerol. The more AU-rich 30 bp dsRNA used in the experiment shown in Figure S2 was assembled from synthetic oligoribonucleotides (Dharmacon), following their 5′-end labeling.

RNA Processing Assays

Unless indicated otherwise, processing of terminally labeled RNAs was performed as described (Zhang et al., 2002), and incubations were for 15 min at 37°C and contained 0.1 pmol of RNA and either 100 ng of Dicer preparation (0.23 pmol; assuming 50% purity of enzyme preparations) or 0.2 ng (3.8 fmol of dimer) of Ec-RNase III. Unless indicated otherwise, cleavage reactions with internally labeled dsRNAs contained 100 ng of Dicer protein and 125 fmol of the substrate, and were incubated for 30 min. Products were analyzed by denaturing 15% PAGE. RNase T1 ladders of the 5′-end-labeled RNAs were 3′-dephosphorylated by treatment with T4 polynucleotide kinase. Native 4% PAGE was performed as described previously (Zhang et al., 2002). Quantification was done using the Storm 860 PhosphorImager (Molecular Dynamics).

Other Procedures

These are described in the Supplemental Material (available on *Cell* website).

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CHAPTER 2

TRBP, a Regulator of Cellular PKR and HIV-1 Virus Expression, Interacts with Dicer and Functions in RNA Silencing

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TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing

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Dicer is a key enzyme involved in RNA interference (RNAi) and microRNA (miRNA) pathways. It is required for biogenesis of miRNAs and small interfering RNAs (siRNAs), and also has a role in the effector steps of RNA silencing. Apart from Argonautes, no proteins are known to associate with Dicer in mammalian cells. In this work, we describe the identification of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) as a protein partner of human Dicer. We show that TRBP is required for optimal RNA silencing mediated by siRNAs and endogenous miRNAs, and that it facilitates cleavage of pre-miRNA *in vitro*. TRBP had previously been assigned several functions, including inhibition of the interferon-induced double-stranded RNA-regulated protein kinase PKR and modulation of HIV-1 gene expression by association with TAR. The TRBP–Dicer interaction shown raises interesting questions about the potential interplay between RNAi and interferon–PKR pathways.

Keywords: Dicer; TRBP; PKR; RNA interference; microRNA

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INTRODUCTION

RNA interference (RNAi)-mediated and microRNA (miRNA)-mediated reactions have emerged recently as important pathways regulating eukaryotic gene expression at various levels. Specificity of these processes is dependent on 20- to 25-nt small interfering RNAs (siRNAs) and miRNAs, acting as guides that recognize sequences of target nucleic acids. To perform their effector function, siRNAs and miRNAs are incorporated into ribonucleoprotein (RNP) complexes, referred to as si- or mi-RISCs (acting post-transcriptionally) or RITS (acting at the chromatin level). Dozens of different proteins have been identified as either essential or regulatory factors for RNAi and miRNA reactions (Tomari & Zamore, 2005). Moreover, it has become increasingly apparent that both pathways intersect with several other cellular processes, such as chromosome segregation (Matzke & Birchler, 2005), RNA editing (Scadden, 2005) and nonsense-mediated degradation (Domeier *et al*, 2000; Kim *et al*, 2005).

MicroRNAs are generated from the genome-encoded precursor hairpins by the sequential action of two ribonuclease (RNase) III-type nucleases, Drosha and Dicer. Dicer is also responsible for the excision of siRNAs from double-stranded (ds)RNA (Kim, 2005; Tomari & Zamore, 2005). However, Dicer is not confined to miRNA and siRNA biogenesis. Each of the two *Drosophila* Dicers, Dcr1 and Dcr2, also seems to be essential for the effector step of RNAi (Tomari & Zamore, 2005). Dcr2, which functions primarily in RNAi, heterodimerizes with a dsRNA-binding domain (dsRBD) protein R2D2 (Liu *et al*, 2003). The resulting complex senses the differential stability of the ends of the siRNA duplex and determines which siRNA strand will enter the RISC (Tomari *et al*, 2004). Recent studies have shown that Drosha and Dcr1, the *Drosophila* Dicer specializing in miRNA biogenesis, function in complexes with dsRBD protein partners DGCR8/Pasha (reviewed by Tomari & Zamore, 2005) and Loquacious (Loqs; Förstemann *et al*, 2005; Saito *et al*, 2005), respectively.

In contrast to *Drosophila*, mammals and *Caenorhabditis elegans* express only a single Dicer protein. Like most other Dicers, mammalian enzymes are large, ~200 kDa, proteins containing ATPase/RNA helicase, DUF283, PAZ domains, two catalytic RNase III domains and a carboxy-terminal dsRBD. Although the biological importance and biochemistry of mammalian Dicer have been intensively studied, little is known about its protein partners. The only proteins known to interact directly with Dicer in mammals are members of the Argonaute (Ago) family, which represent siRNA/miRNA-associating core components

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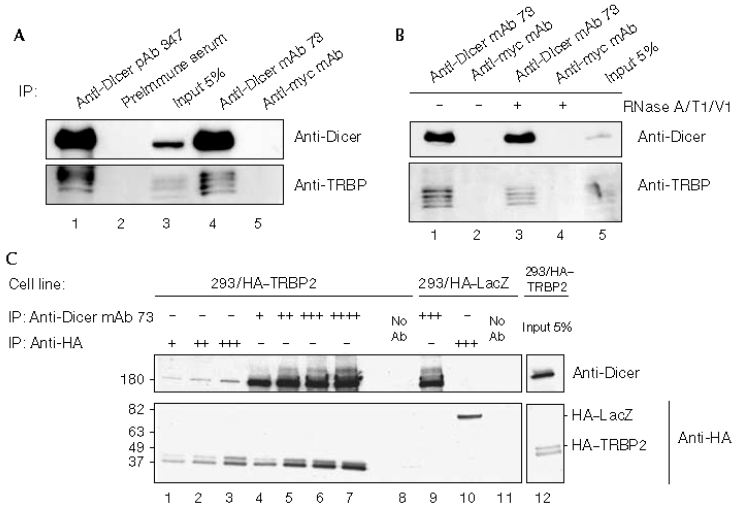

Fig 1 | TRBP and Dicer co-immunoprecipitation. (A,B) Anti-Dicer antibodies (Abs) pull down endogenous TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) in extracts of human embryonic kidney (HEK)293 cells, and co-immunoprecipitation of Dicer and TRBP is not sensitive to ribonuclease (RNase) treatment (B). mAb, monoclonal antibody; pAb, polyclonal antibody. (C) Dicer–TRBP interaction studied with a HEK293 cell line expressing haemagglutinin (HA)-tagged TRBP2. A HEK293/HA–LacZ cell line was used as a control. The identity of the Abs, some at increasing concentrations (+ through + + + +), used for immunoprecipitation (IP) is indicated at the top of the panels. Abs used for western blots are indicated on the right.

of RISCs (reviewed by Tomari & Zamore, 2005). The interaction, involving the RNase III domain of Dicer and a PIWI domain of Argonautes (Tahbaz *et al*, 2004), is probably central to the handover of the products of Dicer catalysis (siRNAs and miRNAs) to Argonautes during RISC assembly.

In this work, we describe the identification of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein; Gatignol *et al*, 1991) as a dsRBD protein partner of human Dicer, and show that TRBP is required for optimal RNA silencing mediated by siRNAs and endogenous miRNAs. TRBP has previously been assigned several functions, including inhibition of the interferon (IFN)-induced dsRNA-regulated protein kinase PKR (Daher *et al*, 2001), modulation of HIV-1 gene expression through its association with TAR (Dorin *et al*, 2003) and control of cell growth (Benkirane *et al*, 1997; Lee *et al*, 2004). A mouse TRBP homologue, Prbp, was shown to function as a translational regulator during spermatogenesis, and mice that have this deletion, in addition to being male sterile, usually died at the time of weaning (Zhong *et al*, 1999). The TRBP–Dicer interaction established in this work raises the possibility of crosstalk between RNAi and IFN–PKR pathways in normal and virus-infected cells.

RESULTS AND DISCUSSION

Dicer and TRBP interact *in vivo* and *in vitro*

We raised monoclonal antibodies (mAbs) against human Dicer (supplementary Fig S1 online). The mAbs 33, 73 and 83, which effectively immunoprecipitate Dicer from extracts of different cultured cells (data not shown), were used to identify proteins associated with Dicer in human embryonic kidney (HEK)293 cells. Proteins retained with either mAbs 33/73/83 or anti-Myc mAb, used as a control, were separated using two-dimensional gel electrophoresis, and spots enriched in Dicer immunoprecipitates were processed for mass spectrometry analysis. One protein reproducibly co-purified with Dicer was identified as TRBP, a protein containing three dsRBDs (supplementary Fig S1 online). Members of the Argonautes family were also among the selected proteins, as were some others, but the reproducibility and significance of their interactions with Dicer were not further investigated (data not shown).

To validate the Dicer–TRBP interaction, we performed co-immunoprecipitation experiments using either extracts from human HEK293 cells or purified proteins. Two anti-Dicer antibodies (Abs), mAb 73 and polyclonal Ab 347, but not the control mAb isotypic with mAb 73, immunoprecipitated endogenous TRBP that was present in HEK293 cells, as shown by western blotting with anti-TRBP Abs (Fig 1A; several forms of TRBP, with apparent molecular masses of 43–46 kDa, are expressed in human cells (see below)). Treatment with RNases digesting both double- and single-stranded RNAs did not eliminate the association (Fig 1B), indicating that the interaction is not mediated by RNA. As immunoprecipitating anti-TRBP Abs were not available, we generated a stable HEK293 cell line, HEK293/HA–TRPB2, expressing the haemagglutinin (HA)-tagged version of the best-studied isoform of TRBP, TRBP2. Co-immunoprecipitation experiments performed with the HEK293/HA–TRPB2 extract and either anti-HA or anti-Dicer Abs showed that each Ab was able to pull down the partner protein (Fig 1C). Further indication that

Mammalian Dicer interacts with the dsRBD protein TRBP
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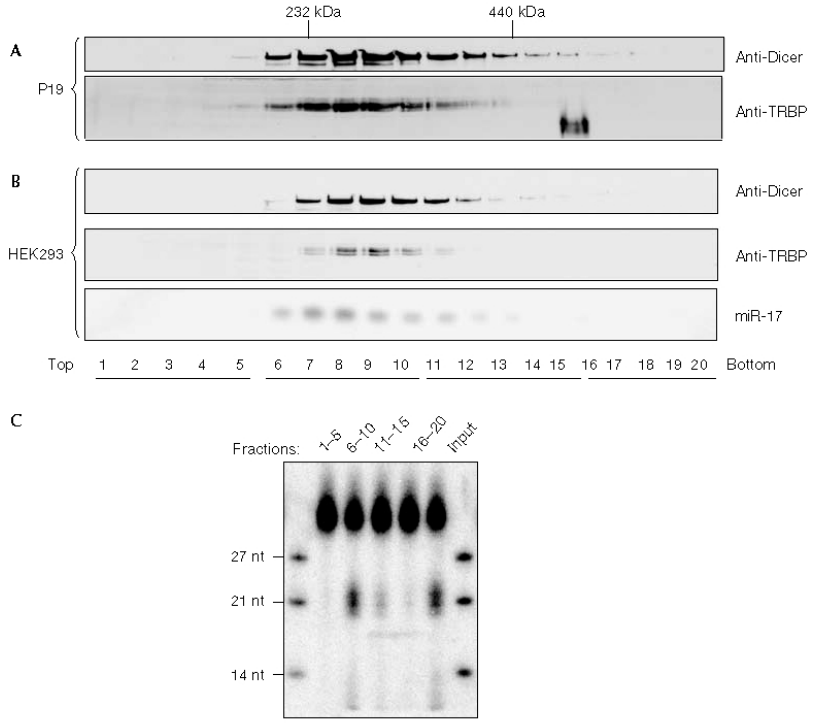

Fig 2 | TRBP and Dicer co-sedimentation on glycerol gradients. (A,B) Sedimentation of cytoplasmic extracts from P19 cells **(A)** and human embryonic kidney (HEK)293 cells **(B)**. Gradient fractions were analysed for Dicer and TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) by western blotting and for miR-17 by northern blotting. **(C)** Activity of fractions analysed in **(B)**, pooled as indicated (top of the panel), in processing 30-bp double-stranded RNA into small interfering RNA.

TRBP and Dicer form part of the same complex was provided by gradient sedimentation experiments. Analysis of cytoplasmic extracts prepared from either human HEK293 or mouse teratoma P19 cells showed that Dicer and TRBP, or their mouse counterparts, co-sediment in a region corresponding to a molecular mass of ~250 kDa (Fig 2A,B). Notably, miR-17, an abundant miRNA in HEK293 cells, was also enriched in this region, as was the activity of processing a 30-bp dsRNA to siRNA (Fig 2B,C). Taken together, the data indicate that Dicer and TRBP interact with each other in mammalian cells.

To find out whether the Dicer–TRBP interaction was direct, we purified both proteins, as recombinant fusions with His_6 and a maltose-binding protein (MBP) from insect cells and *Escherichia coli*, respectively (Fig 3A). The proteins were incubated together and applied either to Protein G–Sepharose beads coated with different Abs or to amylose beads. Dicer mAb 73, but not control anti-HA mAb, effectively pulled down TRBP2. Likewise, TRBP2 retained on amylose beads pulled down Dicer (Fig 3B). The low efficiency of the latter pull-down could be the result of a sterical hindrance caused by the MBP tag or owing to the propensity of TRBP to form homodimers (see below). To eliminate the possibility that proteins co-purifying with either Dicer or MBP–TRBP2 preparations are involved in binding, we studied the Dicer–TRBP interaction in the yeast two-hybrid (2H) assay (Fig 3C). As the budding yeast does not encode TRBP or Dicer homologues, any interaction detected in this system would probably result from direct binding. Plasmids encoding full-length TRBP2, or different regions thereof, fused to the Gal4 DNA-binding domain, and a plasmid encoding Dicer appended to the Gal4 activation domain, as well as several control plasmids, were transformed into yeast. We detected interactions between Dicer and TRBP2 and all its mutants encompassing amino acids 228–366. This region of TRBP includes the dsRBD C domain, suggesting that this domain mediates the interaction (see Conclusions). We also detected TRBP2 interacting with itself, which was consistent with its ability to homodimerize (Daher *et al*, 2001). Taken together, our results indicate that Dicer and TRBP interact directly with each other.

The 45 kDa TRBP2 consists of three dsRBDs. Another previously described TRBP isoform, TRBP1, differs from TRBP2 at the amino terminus (Bannwarth *et al*, 2001; see Fig 3D). By complementary DNA cloning and by inspecting EST databases, we identified another TRBP splice variant, potentially encoding a TRBP3 isoform, which would miss the C-terminal dsRBD (Fig 3D). Interestingly, one of the three isoforms of Loqs, the probable

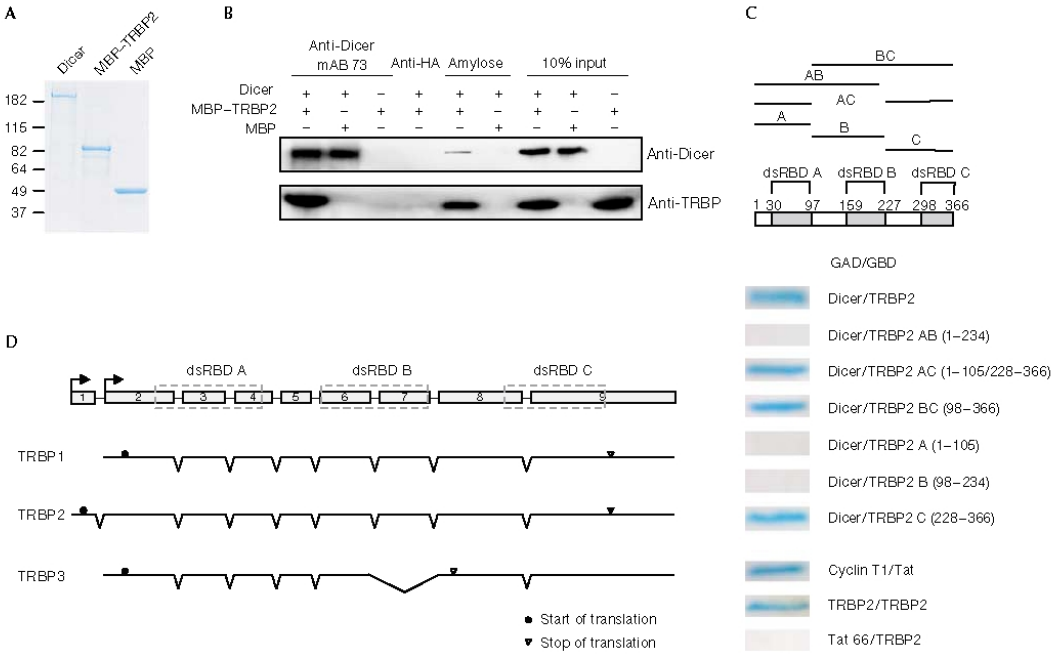

Fig 3 | Interaction of TRBP with Dicer studied with purified proteins (A,B) and in the yeast two-hybrid system (C), and schematic representation of different TRBP transcripts expressed in human cells (D). (A) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of indicated purified recombinant proteins. MBP, maltose-binding protein. (B) Purified TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) and Dicer interact with each other. Added proteins, antibodies (Abs) and amylose beads are indicated at the top. Abs used for western blotting are shown on the right. HA, haemagglutinin; mAb, monoclonal antibody. (C) Dicer and TRBP interact in a yeast two-hybrid (2H) assay through the carboxy-terminal domain of TRBP. Top: scheme of TRBP2 and its fragments included in 2H constructs. Double-stranded RNA-binding domains (dsRBDs) are shown as grey boxes. Bottom: β-galactosidase staining diagnostic of the interaction between proteins expressed as fusions with either Gal4 DNA activation domain in pGADGH (GAD) or Gal4 DNA binding domain in pGBT9 (GBD). Cyclin T1/Tat and TRBP2/TRBP2 are positive controls. Tat/TRBP2 is a negative control. (D) Scheme of the TRBP gene and its encoded transcripts. Intron positions and exon regions encoding three dsRBDs are indicated. Two alternative transcription starts, and translation initiation and stop codons, are indicated by arrows, circles and triangles, respectively. Skipping of exon 7 in TRBP3 causes translation to terminate in exon 8.

Drosophila homologue of TRBP, is also devoid of the C-terminal dsRBD (Förstemann *et al*, 2005). The biological function of individual TRBP variants remains unknown. The alignment of TRBP2 with dsRBD Dicer protein partners from other species and with a TRBP-related mammalian protein PACT is shown in supplementary Fig S3A online. High sequence conservation of the C-terminal dsRBD in Loqs, TRBP2 and PACT (supplementary Fig S3B online) suggests that this domain has a function distinct from two other dsRBDs (see below).

TRBP is required for miRNA and siRNA silencing

To assess the potential role of TRBP in RNAi and miRNA pathways, we generated stable HEK293T-REx cell lines, in which the expression of TRBP is knocked down by RNAi. In cell lines 293/TRBPkd1 and 293/TRBPkd2, the expression of stably integrated short hairpins targeting different regions in TRBP messenger RNA is controlled by a pol III promoter with tetracycline (Tet) operator sites. Real-time PCR and western analyses indicated that TRBP expression was about fivefold lower at both mRNA and protein levels. In either cell line, partial decrease of the protein had already occurred in the absence of Tet induction, indicating some leakiness of the system (Fig 4A; supplementary Fig S2 online). TRBP depletion had no appreciable effect on cell growth (data not shown).

We compared the miRNA-precursor-processing activity of extracts prepared from different cell lines (Fig 4B). Extracts from either TRBPkd cell line processed pre-let-7 RNA less efficiently than extracts from control cells. The decrease in activity was not due to destabilization of Dicer, as its level was similar in control and TRBPkd cells (Fig 5B). Despite extracts from TRBPkd cells being deficient in pre-miRNA processing, steady levels of several miRNAs in these cells were not significantly different from control HEK293 cells, and there was no apparent accumulation of pre-miRNAs (Fig 4C; data not shown). Notably, as in the case of TRBP,

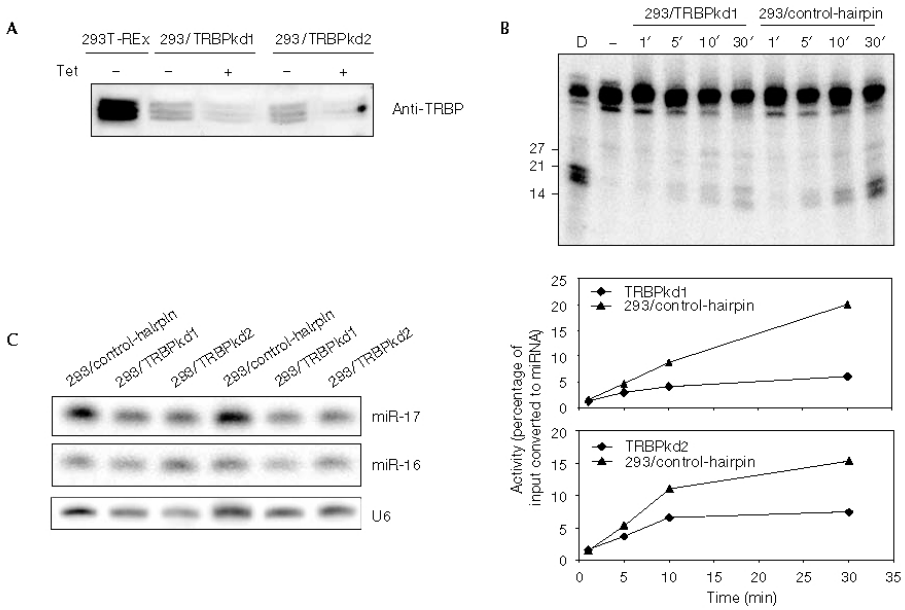

Fig 4 | Depletion of TRBP affects pre-microRNA processing *in vitro* but has no effect on accumulation of mature microRNAs *in vivo*. (**A**) Levels of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) in 293/TRBPkd1/2 and human embryonic kidney (HEK)293T-REx control cell lines after treatment with tetracycline (Tet) for 72 h as indicated. Anti-TRBP antibody was used for western blotting. (**B**,**C**) Effect of TRBP knockdown on processing of pre-let-7 RNA *in vitro* (**B**) and on accumulation of mature microRNAs (miRNAs) *in vivo* (**C**). (**B**) Processing of pre-let-7 RNA was assayed in extracts from 293/TRBPkd1, 293/TRBPkd2 and 293/control-hairpin cell lines. Phosphorimage of 293/TRBPkd1 (upper panel) and quantification of data for both TRBPkd cell lines (bottom panels) are shown. (**C**) Total RNA isolated from TRBPkd cell lines, from two independent cultures and control cell lines was analysed by northern blot using probes specific for indicated RNAs. Quantification of data from four independent northern blots showed no significant differences in miR-16 and miR-17 levels between TRBPkd and control cultures. A representative experiment is shown. Similar results were obtained for miR-19B and let-7 miRNAs (data not shown).

depletion of Loqs in *Drosophila* S2 cells had no principal effect on mature miRNA levels although extracts of Loqs knockdown cells were deficient in pre-miRNA processing. However, in contrast to TRBP, depletion of Loqs resulted in accumulation of pre-miRNAs in S2 cells (Förstemann *et al*, 2005; Saito *et al*, 2005).

We used the miRNP-mediated mRNA-reporter-cleavage assay to find out whether depletion of TRBP had an effect on the activity of endogenous miRNPs in HEK293 cells. TRBPkd and control cells were transiently transfected with constructs expressing either control *Renilla* luciferase (RL) reporter mRNA or a reporter harbouring the site perfectly complementary to miR-17 in its 3′ untranslated region. In control cells, insertion of the miR-17 site repressed RL expression by ~80%. However, in TRBPkd cells, the miRNA-mediated inhibition was about threefold less pronounced (Fig 5A), indicating that TRBP is required for either the assembly or functioning of miRNPs.

To investigate whether TRBP has a role in the RNAi reaction mediated by exogenous siRNA, we determined the efficiency of the lamin A/C RNAi in TRBPkd and various control cells. The siRNA treatment had a strong effect on lamin A/C level in parental HEK293T-REx cells or cells stably expressing a control hairpin. However, lamin A/C depletion was largely abolished in TRBPkd cells. Similar suppression of the lamin A/C knockdown was observed in a HEK293 cell line in which Ago2 was depleted by expression of a specific hairpin (Fig 5B).

Taken together, our data indicate that TRBP is primarily required for the assembly and/or functioning of si- or mi-RISCs in mammalian cells, but it may also facilitate the cleavage of pre-miRNAs by Dicer. The apparent discrepancy between the effect of TRBP knockdown on pre-miRNA processing in cells and cell extracts is readily explained by incomplete depletion of the protein, allowing for the manifestation of processing deficiency *in vitro* but not *in vivo*. It is worth noting that, as in the case of *Drosophila* Loqs (Förstemann *et al*, 2005; Saito *et al*, 2005) but in contrast to R2D2 (Liu *et al*, 2003), depletion of TRBP did not lead to appreciable destabilization of Dicer (Fig 5B).

CONCLUSIONS

Our findings that mammalian Dicer forms a complex with a dsRBD protein TRBP add support to the idea that large RNase III-type Drosha and Dicer nucleases generally require dsRBD protein partners for their function. *Drosophila* R2D2 and Loqs are two *Drosophila* dsRBD proteins that work in conjunction with Dcr2 and Dcr1, acting in RNAi and miRNA pathways, respectively

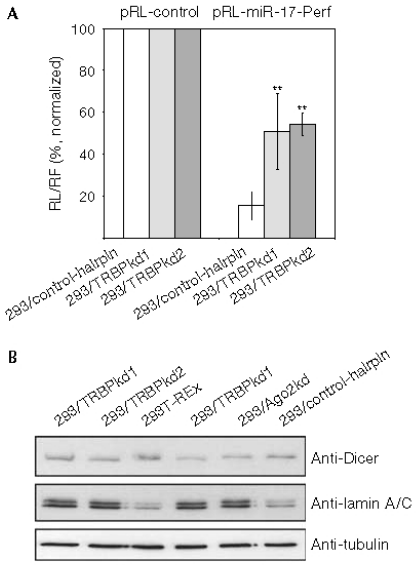

Fig 5 | Effect of TRBP depletion on RNA silencing. Depletion of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) decreases the efficiency of RNA interference mediated by the endogenous microRNA miR-17 (**A**) and by transfected anti-lamin A/C small interfering RNA (**B**). Cell lines used for analysis are indicated. In (**A**), activities of *Renilla* luciferase (RL)-miR-17-Perf reporter in every cell line are expressed in relation to activities of RL-control reporter (set as 100%). Values are means ± s.d. of four transfections (**$P<0.01$). Similar results were obtained in several independent experiments. Antibodies used for western blotting in (**B**) are indicated on the right.

(Liu *et al*, 2003; Förstemann *et al*, 2005; Saito *et al*, 2005). The R2D2–Dcr2 association is required for asymmetric loading of siRNAs into RISC (Tomari *et al*, 2004), whereas Loqs and Dcr1 are essential for efficient pre-miRNA processing, and also participate in gene silencing that is triggered by artificial dsRNA hairpins and endogenous *Supressor of Stellate* repeats (Förstemann *et al*, 2005; Saito *et al*, 2005). The observation that TRBP is required for efficient cleavage of pre-miRNA *in vitro* and for the function of RISC programmed with either endogenous miRNA or transfected siRNA in cells indicates that TRBP combines at least some functions that are performed separately by R2D2 and Loqs in *Drosophila*. However, it should be noted that TRBP is structurally more related to Loqs than to R2D2 (supplementary Fig S3 online; Förstemann *et al*, 2005). We investigated, using both cell extracts and recombinant proteins, whether Dicer and TRBP are involved in sensing the thermodynamic stability of the 5′ ends of the siRNA strands in the same way as Dcr2 and R2D2. These experiments, using 5-iodo-U-modified siRNAs, have not produced conclusive results (L.J., unpublished results).

After the submission of this work, another work describing the TRBP–Dicer partnership has been reported (Chendrimada *et al*, 2005). Two observations described in the Chendrimada *et al* paper are in disagreement with our results. First, they found that depletion of TRBP resulted in a decrease of steady-state levels of miRNAs, whereas in our analysis the miRNA content was not significantly changed. Second, in contrast to our findings, Chendrimada *et al* report that knockdown of TRBP causes destabilization of Dicer, and vice versa. However, we note that the analysis of TRBP and Dicer levels was carried out not with total extracts from cells depleted in either protein but with Argonaute (Ago2) immunoprecipitates. Hence, it is possible that depletion of Dicer or TRBP affects the ability of the partner protein to form a complex with Ago2 rather than destabilizing the protein. This interpretation would be consistent with a relatively mild phenotype of mice with a knockout of Prbp, the mouse homologue of TRBP (Zhong *et al*, 1999), contrasting with the lethal phenotype of the Dicer knockout (Bernstein *et al*, 2003). It would also support our observations that the efficient depletion of Dicer in HEK293 cell lines has no principal effect on the level of TRBP (A.H., K. Tang & W.F., unpublished results).

Another three-dsRBD protein, PACT, 42% identical to TRBP, is expressed in mammals. In contrast to TRBP, which inhibits PKR, PACT (or its mouse homologue Rax) has a stimulatory effect on PKR (Gupta *et al*, 2003; Bennett *et al*, 2004, and references therein). The effects of TRBP and PACT on PKR activity are mediated by the C-terminal dsRBDs, which are devoid of detectable dsRNA-binding properties (Gupta *et al*, 2003, and references therein). In addition to effects on PKR, the C-terminal domains of PACT and TRBP can mediate heterodimerization of both proteins and also homodimerization of PACT (Hitti *et al*, 2004; G. Laraki & A.G., unpublished results). The apparent involvement of the C-terminal TRBP region in association with Dicer (Fig 3C) raises the possibility that RNAi/miRNA and PKR pathways are subject to reciprocal regulation by a rather complex network of protein–protein interactions. As both RNAi and IFN–PKR pathways have a role in antiviral responses, communication between them would not be surprising. In the future, it would be interesting to find out whether Dicer also associates with PACT, and how these protein interactions affect RNA silencing and other defence pathways in normal and virus-infected cells. The latter question is particularly interesting in the light of a recent report that the HIV-1 TAR-binding protein Tat functions as an RNAi suppressor, possibly compromising the activity of Dicer (Bennasser *et al*, 2005).

METHODS

Co-immunoprecipitations. Anti-Dicer mAbs 33, 73 and 83 and control mAbs were crosslinked to Protein G–Sepharose 4 Fast Flow (Amersham Biosciences, Little Chalfont, UK) and used to purify Dicer complexes from HEK293 cytoplasmic extracts (S10). Co-immunoprecipitates were washed five times with lysis buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.5% NP-40, 2.5 mM $MgCl_2$) and analysed by liquid chromatography tandem mass spectrometry (LC-MSMS) and western blot.

293/TRBPkd1, 293/TRBPkd2 and 293/control-hairpin cell lines. Plasmids pTER-TRBPsh1, pTER-TRBPsh2 and pTER-control-hairpin were co-transfected with a puromycin resistance plasmid into HEK293T-REx cells (Invitrogen, Carlsbad, CA, USA) to generate stable cell lines.

Other procedures. Detailed methods can be found in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

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CHAPTER 3

RNA binding properties of human Dicer dsRBD

Introduction

Five different domain types are present in human Dicer: helicase/ATPase, DUF283, PAZ, RNase IIIa and b, and dsRBD. Functions of helicase and DUF domains remain to be identified. Majority of biochemical experiments were targeted at elucidating the role of RNase III and PAZ domain in dsRNA cleavage. dsRBD, the double stranded RNA binding domain of Dicer, was shown to bind dsRNA previously (Provost et al., 2002) and was generally thought to mediate unspecific binding of Dicer to dsRNA, stabilizing the enzyme-substrate interaction. In the course of this work it was determined that human Dicer deleted for dsRBD is still active in both dsRNA cleavage and pre-miRNA processing, however its activity was reduced 2-3 fold (Zhang et al., 2004).

Recently, new roles different than simple dsRNA binding have emerged for dsRBD. There is structural evidence and also experimental data which support the notion that individual dsRBDs within a protein can specifically recognize and discriminate between different RNAs *in vivo*. Examples include dsRBDs of Staufen recognizing tetraloops (Ramos et al., 2000), dsRBD2 of Staufen that is responsible for microtubule-dependent localization of oskar mRNA whereas dsRBD5 is needed for its translational control (Micklem et al., 2000), dsRBDs of *Xenopus laevis* ADAR1 that target the enzyme to different transcriptionally active lampbrush chromosomal loops (Doyle and Jantsch, 2003) and dsRBD2 of ADAR1 and dsRBD2 of XLRBPA that have recently been shown to specifically recognize different imperfectly double stranded substrates identified by the SELEX procedure (Hallegger et al., 2006).

dsRBD roles reach beyond RNA binding. It has been shown that the third dsRBD of human ADAR-1 has an atypical Nuclear Localization Signal (NLS) that overlaps entirely with the dsRBD domain. Moreover, NLS activity of dsRBD3 does not depend on RNA binding, showing a new function of that domain (Eckmann et al., 2001). DsRBD is also involved in mediating protein-protein interactions. In case of TRBP and its ortholog PACT it has been shown that their third dsRBD is important for formation of homodimers (Daher et al., 2001). In addition, PKR activation by PACT and inhibition by TRBP is mediated via their dsRBD3 (Gupta et al., 2003).

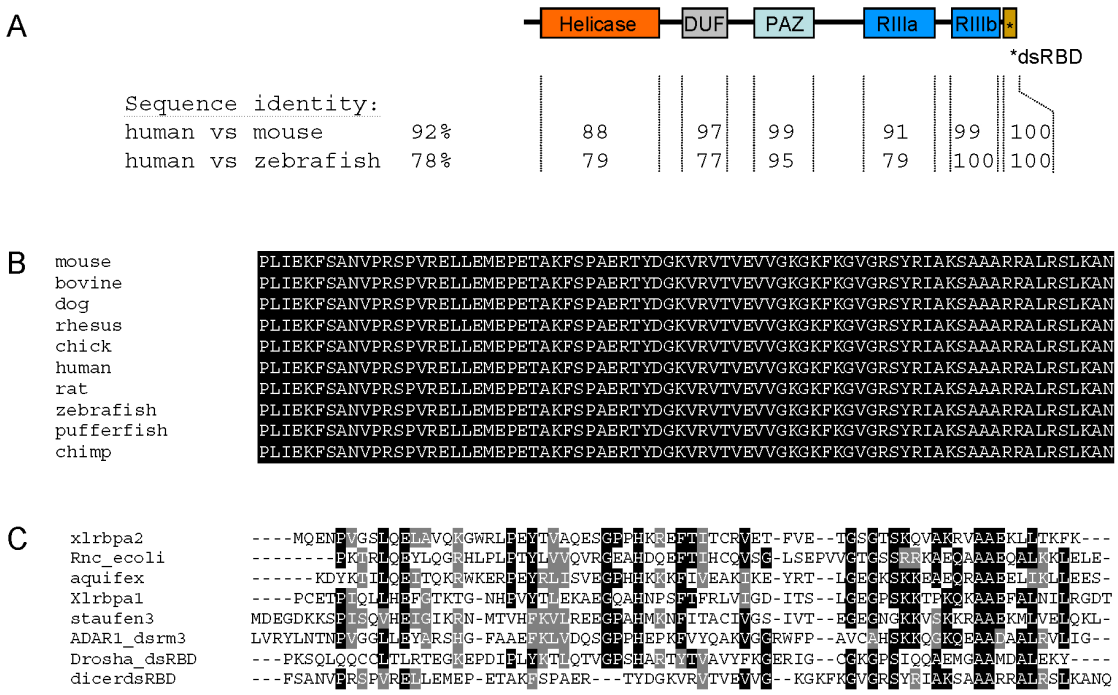

Fig. 1. DsRBD conservation. (A) Conservation of individual domain sequences between human, mouse and zebrafish Dicers. The percent of identity of the whole Dicer sequence is also shown on the left. (B) Alignment of vertebrate Dicer dsRBDs. (C) Alignment of human Dicer dsRBD with other dsRBD sequences.

Human Dicer dsRBD is the most conserved domain within vertebrate Dicers (Fig. 1A and 1B), however, its sequence differs quite significantly from the general dsRBD consensus (Fig. 1C). We wanted to characterize dsRNA binding properties of human Dicer dsRBD. Dicer dsRBD was shown to bind dsRNA previously (Provost et al., 2002), however the experiments were performed using a fusion protein encompassing nearly half of the RNase IIIb and the dsRBD. In addition no quantitative data or substrate length preferences were provided.

Results

dsRBD of human Dicer has a higher affinity for longer dsRNAs and exhibits very low siRNA binding activity

To characterize the nucleic acid binding properties of dsRBD, we performed gel mobility shift assays with purified recombinant dsRBD fused to GST and expressed in *Escherichia coli* (Fig. 2A) using dsRNA substrates of different lengths. ^{32}P-labeled dsRNA was incubated with increasing concentrations of dsRBD and subsequently resolved by native polyacrylamide gel electrophoresis, enabling the visualization of protein/RNA complexes. We tested the ability of dsRBD to bind dsRNA of different lengths: 130, 70, 50, 30 and 19-bp dsRNA. The 30-bp and 19-bp long dsRNAs tested contained two nucleotide 3' overhangs, thus 19-bp long dsRNA mimicked an siRNA.

As a control we used GST tagged second dsRBD of *Xenopus laevis* dsRNA binding protein A (XLRBPA) purified in the same way. The XLRBPA dsRBD was previously shown to bind dsRNA (Krovat 1996). Both Dicer dsRBD (referred to as D-dsRBD) and XLRBPA dsRBD (XL-dsRBD) readily formed stable complexes with 130, 70, 50 and 30-bp dsRNA (Fig. 2B). XL-dsRBD was also able to bind 19-bp long dsRNA, however D-dsRBD shown very little affinity for this siRNA-like molecule (Fig. 2C). GST alone did dot bind any of the RNA tested (Fig. 2C and data not shown).

We have determined dissociation constants K_d for interaction of both dsRBDs with different substrates (Table 1). Generally, XL-dsRBD binds dsRNA

with ~2 orders of magnitude higher affinity than D-dsRBD, and longer substrates are bound better by both dsRBDs.

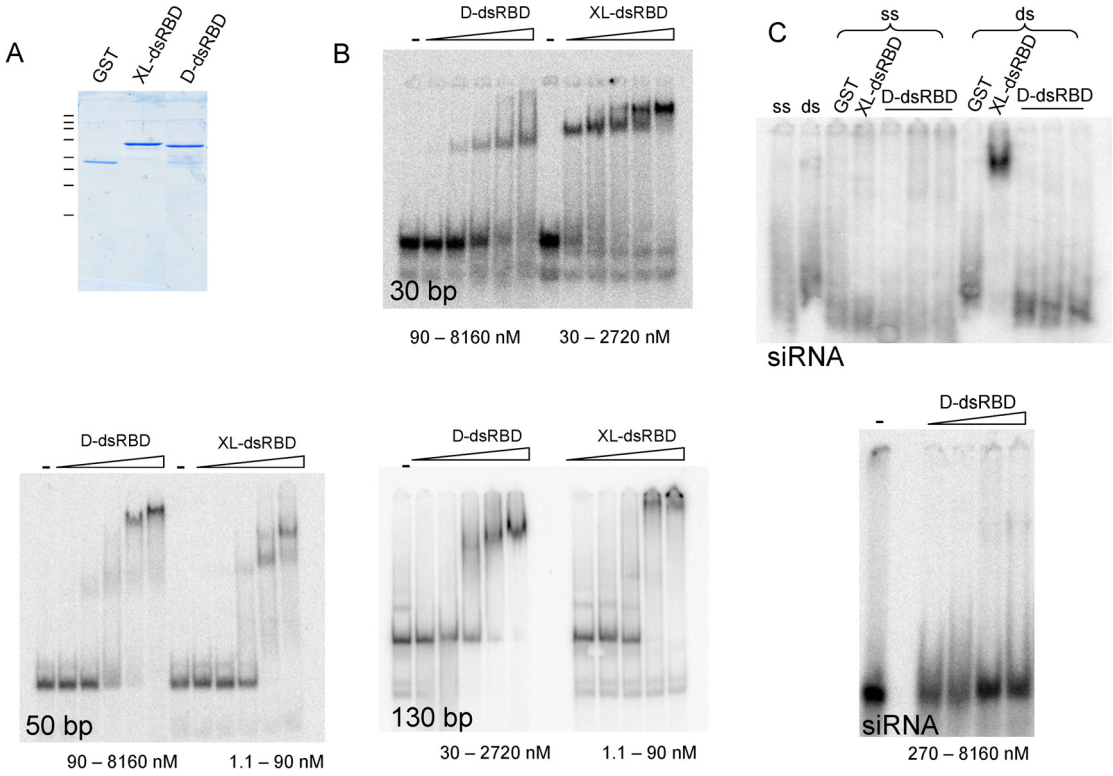

Fig. 2. DsRNA binding properties of Dicer dsRBD. (A) SDS-PAGE of *E. coli* expressed and purified recombinant GST-fusion dsRBD and XLRBPA dsRBD2. (B) Dicer dsRBD binds 30-, 50- and 130-bp-long dsRNAs as assayed by native PAGE. The concentration range of the protein used is indicated. (C) dsRBD of Dicer does not bind siRNA duplex. ssRNA of 21 nt is used as a control. dsRBD of Dicer is used in increasing concentrations – 900, 2720 and 8160 nM.

RNA (bp)	K_d (nM)	
	Dicer dsRBD	XLRBPA dsRBD2
19+2 (a) 19+2 (b) 21 (blunt)	>8000	~13
30	332	11.5
50	251.5	9.3
130	172	5.5

Table 1. Dissociation constants K_d for interaction of Dicer dsRBD and XLRBPA dsRBD2 with different substrates

Design of dsRNA binding-deficient mutants of human Dicer dsRBD

Since the general dsRDB consensus is poorly conserved in Dicer dsRBD, it is hard to predict which residues could directly contribute to dsRNA binding. Based on XLRBPA mutation data (Krovat and Jantsch, 1996) and the its crystal structure in a complex with dsRNA (Ryter and Schultz, 1998) we chose 9 residues to mutate within D-dsRBD and created point-mutated versions of the domain, designated mut1 to mut9 (Fig. 3A). Mut1, 2, 8 and 9 were expressed in insoluble form and failed to purify, most likely due to a folding defect caused by the introduced amino acid substitutions. Other mutants were soluble (Fig 3B) and were tested for dsRNA binding using 50- and 130-bp long substrates. Binding of mut4 was unaffected (data not shown), while mut3, 5, 6 and 7 showed reduced RNA binding activity (Fig. 4A). Based on analysis of complexes formed with different protein concentrations with dsRBD, resolved by native PAGE, we estimated that the binding of mut7, showing the strongest effect, was reduced 3 to 9 fold. We have also purified multiple mutants. Double mutant combined mut3 and 7, quadruple mutant was a combination of all four single mutations. Both, the double and the quadruple mutants, showed further reduced binding activities (Fig. 4B).

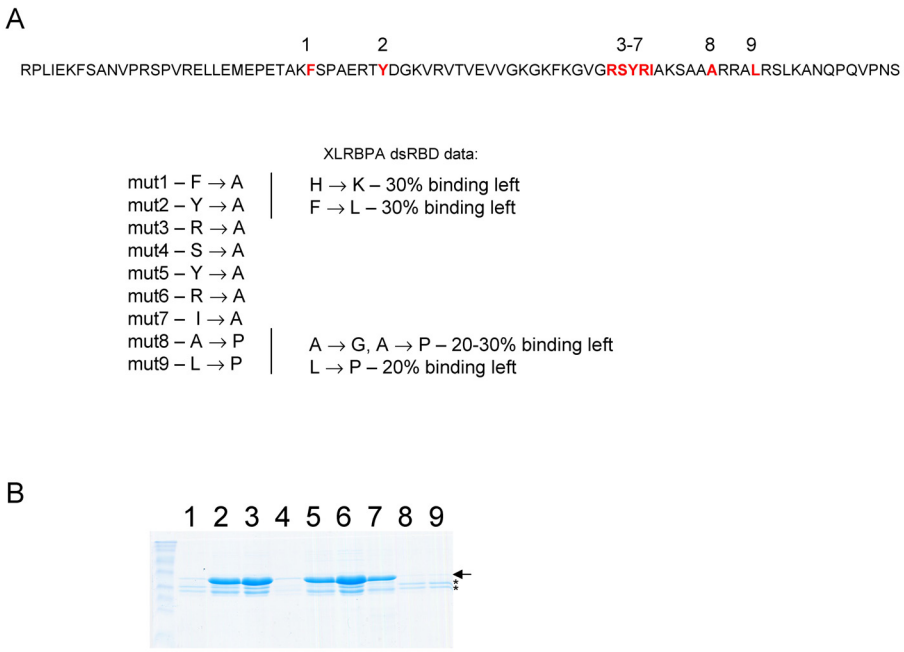

Fig. 3. Summary of the dsRBD mutants generated. (A) Sequence of human Dicer dsRBD. The mutated residues are indicated in red. XLRBPA mutagenesis data are provided for equivalent mutants. (B) SDS-PAGE of purified mutated proteins. Full length GST-dsRBD is indicated by an arrow. Asterisks mark contaminating bands.

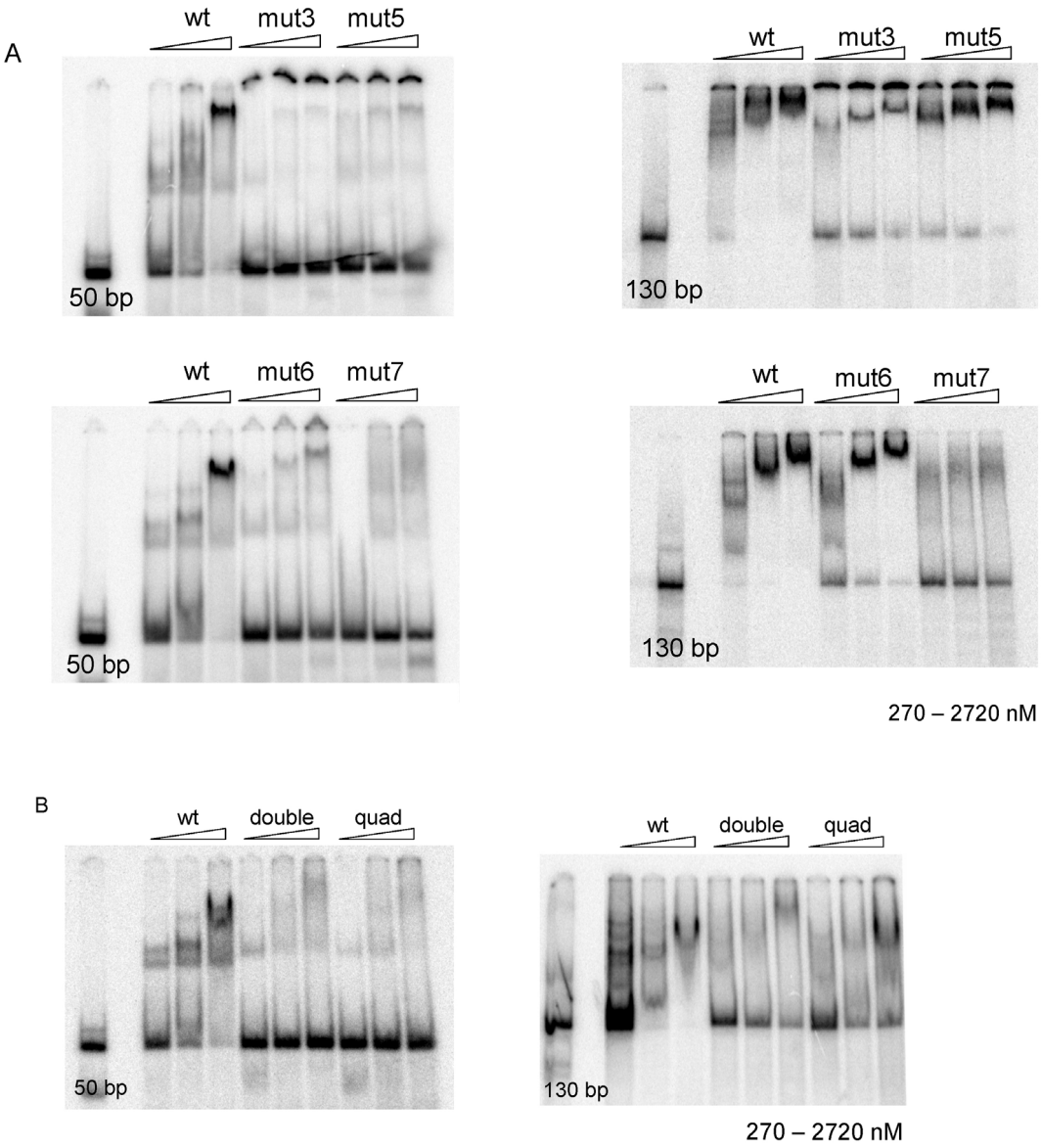

Fig. 4. 50 and 130 bp long dsRNA binding by dsRBD point mutants (A) and multiple mutants (B) as assayed by native PAGE. Concentrations of proteins used are indicated.

Discussion

The observation that dsRBD of human (and because of very high degree of conservation possibly also other vertebrates) Dicer very weakly binds RNAs of the 19-bp has several implications. During dsRNA and pre-miRNA cleavage reaction dsRBD appears to stabilize the binding of substrate. This is supported by the observation that activity of Dicer-ΔdsRBD is reduced ~2-3 fold in processing of both pre-let7 miRNA and dsRNA (Zhang et al., 2004). However, after the cleavage dsRBD would not contribute to the binding of the siRNA/miRNA product. Since Dicer alone is shown to bind siRNA (Pellino et al., 2005), binding activity would come from other protein domains able to bind RNA, like PAZ and the catalytic core of RNase IIIa and IIIb. The binding is most likely needed for handing the siRNA over to RISC. Consecutively, during Dicer function at the effector step dsRBD would not participate in binding, perhaps lowering the affinity of the enzyme for its cleavage product and favoring siRNA/miRNA release or handing over to downstream components, such as Ago proteins (Fig. 5). It is also conceivable that like in *Drosophila*, mammalian Dicer participates in determination of siRNA/miRNA polarity before siRNA/miRNA enters RISC. Since Dicer always approaches the substrate from the dsRNA end, in some of the situations siRNA/miRNA 'flipping' would have to occur to ensure incorporation of the guide strand.

Recently RNA-binding properties of RDE-4 were also investigated (Parker et al., 2006). It was found that purified RDE-4 binds with higher affinity to long dsRNA. This is in agreement with our observations on Dicer dsRBD and

XLRBPA dsRBD2 and suggests that the better binding of longer substrates is an intrinsic property of dsRBD. However our data show, that unlike XLRBPA and RDE-4, Dicer dsRBD binds substrates of the siRNA size with very low affinity.

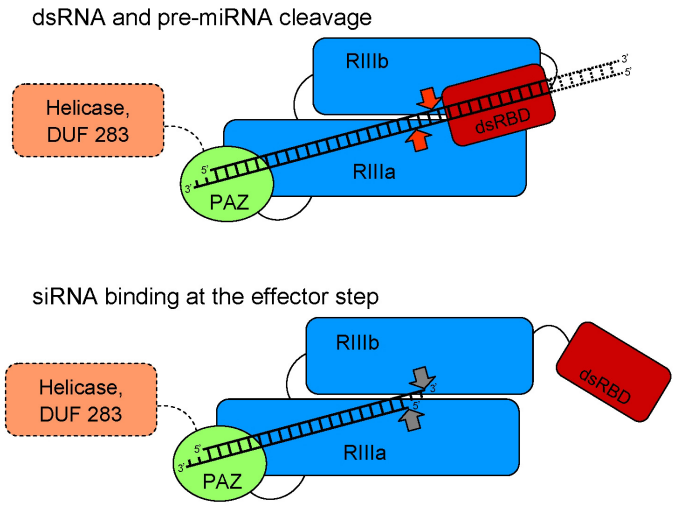

Fig. 5. Schematic representation of two different dsRNA binding modes of human Dicer. During dsRNA and pre-miRNA processing the dsRBD contributes to binding and stabilizes the substrate. When Dicer remains associated with siRNA after the cleavage, dsRBD does not participate in binding, potentially lowering the enzyme affinity in order to facilitate siRNA release or its function at the effector step.

We have found that dsRBD of Dicer acts as an atypical Nuclear Localization Signal (NLS) (M. Doyle, unpublished results). To determine whether the NLS activity of dsRBD depends on RNA binding we have characterized 6 different mutants that were defective in dsRNA binding. The ability of the dsRBD of human Dicer to localize to the nucleus appears to be RNA-independent (M. Doyle, unpublished data).

Three-dimensional structure of XLRBPA dsRBD2 has been determined (Ryter and Schultz, 1998). It was crystallized in a complex with dsRNA duplex formed by annealed self-complementary 10-nt long oligonucleotide. Within the

crystals, the 10 bp dsRNA helices stack end-to-end as a pseudo-continuous helix. dsRBD2 spans a total of 16 bp of RNA. Other solved structures of different dsRBDs align well with the *X. laevis* domain indicating that the minimal length of dsRNA for efficient binding could be similar. This includes solution structures of human PKR (Nanduri et al., 1998), mouse Staufen (Bycroft et al., 1995), human TRBP2 (pdbid 1di2), and *S. cerevisiae* Rnt1p (Leulliot et al., 2004), and also crystal structures of *Aquifex aeolicus* and *Thermatoga maritima* RNase III (Blaszczyk et al., 2004 and pdb id 1o0w). Our finding that Dicer dsRBD very weakly binds substrates of 19 bp suggests that this dsRBD possibly adapts different conformation than other known dsRBDs.

Determination of the 3D structure of dsRBD of Dicer would certainly prove to be very informative both for greater understanding of the mechanism of dsRNA recognition by dsRBD and to explain its role within the full-length Dicer and in dsRNA processing.

Materials and methods

Expression constructs.
Fragment encoding dsRBD of human Dicer (residues 1839-1915) was amplified by PCR using pBS-Dicer as a template (Zhang et al., 2002) and cloned into BamHI/HindIII sites of pGEX expression vector. Plasmid expressing GST-tagged dsRBD2 of XLRBPA was described previously (Krovat and Jantsch, 1996).

Protein expression and purification
GST-fusion proteins were expressed in E. coli BL21-CodonPlus(DE3)-RIPL cells (Stratagene), purified using GS-sepharose (Amersham) according to manufacturer's instructions, dialysed against buffer containing 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 50% glycerol and stored in -20 °C. Purity of proteins was analyzed by 15% PAGE. Protein concentration was determined by Bradford method with BSA as a standard.

Mutagenesis of dsRBD
Mutagenesis was performed using Quikchange site directed mutagenesis system (Stratagene) according to manufacturer's instructions.

Preparation of RNA substrates
The internally ^{32}P-labeled 30-bp, 50-bp, 70-bp and 130-bp dsRNA were prepared as described before [Zhang 2002]. RNAs were synthesized by the T7 polymerase in vitro transcription, using the Ambion T7 MaxiScript transcription kit and [α-^{32}P]UTP. After transcription, samples were RNA purified by denaturing 8% PAGE. Complementary RNA strands were annealed at 95 °C for 3 min in 20 mM NaCl, transferred to 75 °C, and then slowly cooled down to 20 °C.
The 5'-end-labeling of siRNA, using T4 polynucleotide kinase and either [γ-^{32}P]ATP or cold ATP, was carried out as described by Sambrook et al., (1989).

Analysis dsRBD-dsRNA complexes by native PAGE

10-microliter reactions containing 300 pM-labeled dsRNA and varying protein concentrations were incubated for 30 min at 37 °C in buffer containing final concentration of 30 mM Tris pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$ and 25% glycerol. Complexes were analyzed on 5% native polyacrylamide gels with acrylamide/bis-acrylamide ratio on 19:1. Gels were electrophorezed in 1xTBE buffer at 4 °C, dried and quantified by Phosphoimager. Fraction bound was calculated using Molecular Dynamics Image Quant 5. Radioactivity corresponding to $dsRNA_{free}$ and $dsRNA_{total}$ (total radioactivity in entire lane) was quantified. Apparent dissociation constants (Kd) were calculated by fitting the experimental data by nonlinear least-squares regression to the single-site binding isotherm:

% free RNA = Kd[app]/(Kd[app] + [protein]).

From this equation, the apparent Kd corresponds to the protein concentration at which half of the RNA is bound. Fitting of the data was done using Prism 5 (GraphPad).

Sequences of RNAs used:

siRNA(a):

upper strand: 5’ GCAGCACGACUUCUUCAAGUU 3’

lower strand: 5’ CUUGAAGAAGUCGUGCUGCUU 3’

siRNA(b):

upper strand: 5’ GUCACAUUGCCCAAGUCUCUU 3’

lower strand: 5’ GAGACUUGGGCAAUGUGACUU 3’

21blunt:

upper strand: 5’ AAGUCACAUUGCCCAAGUCUC 3’

lower strand: 5’ GAGACUUGGGCAAUGUGACUU 3’

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GENERAL DISCUSSION

The aim of this work was to further biochemically characterize the activity of the purified human Dicer, a key enzyme involved in RNAi pathway, to get an insight into the dsRNA/pre-miRNA cleavage mechanism, and also to characterize the heterodimeric Dicer/TRBP complex functioning in the RNAi/miRNA pathway.

The X-ray structure of the bacterial RNase III has been solved, and a model of how this enzyme binds to dsRNA and processes it into ~11 bp products was proposed (Blaszczyk et al., 2001). Since Dicer belongs to the RNase III superfamily, the bacterial RNase III structure and its activity model suggested a possible mechanism of dsRNA cleavage by Dicer. Moreover, a model was proposed to explain the size difference between the products of RNase III (~11-bp) and Dicer (~22-bp). The model was not conclusive and did not address several important issues. The proposed catalytic residues E37 and E64 were not involved in co-ordination of a metal ion. The size difference was speculatively explained by the fact that one of the evolutionarily conserved putative catalytic residues in Dicer is changed from glutamate to proline (Proline 1729 in human Dicer, P64 according to *A. aeolicus* numbering used throughout this discussion). This substitution however should only partly incapacitate the cleavage site, still allowing the remaining residues D44 and E110 to cleave one of the dsRNA strands. Also, the model was only tested in an indirect assay by assessing the Ec-RNase III dependent expression of the λN-lacZ reporter *in vivo* (Blaszczyk et al., 2001).

We have previously established the *in vitro* processing assay with a purified Dicer (Zhang et al., 2002) and we have used this system to investigate

the mechanism of dsRNA cleavage by Dicer and in parallel by Ec-RNaseIII. To address this problem, the putative catalytic residues in the two Dicer RNase III domains, as based on the bacterial RNase III model, were mutated to yield single amino acid substitutions or combinations thereof.

Analysis of Dicer mutants showed that Dicer processes dsRNA by a mechanism different from the one in the proposed model. Mutation of residues in positions 37 and 64 in both RNase III domains did not lead to an appreciable difference in enzyme activity as compared with the wild type enzyme. Results obtained with mutants of the *E. coli* RNase III confirmed the effect seen in Dicer. Mutations of E37 and E64 to alanine caused the enzyme to increase its K_M without affecting the V_{max}, suggesting that these residues are not directly involved in catalysis but may contribute to substrate binding.

These data indicated that the previously proposed model is not correct. Based on the results of RNase III and Dicer mutagenesis, we proposed new models for the substrate cleavage by both the E. coli RNase III and Dicer. In the bacterial RNase III model, the dsRNA substrate is rotated by approximately 30 degrees with regard to its position in the old model of Blaszczyk et al. (2001). In the new model, residues in positions 37 and 64, previously proposed to be important for the catalysis, play no role in the cleavage. For Dicer, an intra-molecular pseudo dimer model is proposed. In this model, Dicer RNase IIIa and RNase IIIb domains form together one compound catalytic center. Further support for this model came from the results of gradient sedimentation which showed that Dicer behaves as a monomer rather than as a dimer. Our results

have recently been fully supported by the determination of crystal structure of the *Giardia intestinalis* Dicer by the laboratory of Jennifer Doudna (Macrae et al., 2006). The RNase III domains form the catalytic domain and the PAZ domain is directly connected to the RNase IIIa domain by a long 'connector' alpha helix which may act as a measuring ruler determining the product length. Based on the crystal structure, the role for the conserved 'domain of unknown function 283' (DUF283) was proposed (Fig. 4, Introduction). Low but significant sequence homology between the N-terminal domain of *Giardia* Dicer and DUF283 suggests that DUF283 forms a platform structure similar to that of *Giardia* Dicer also in Dicers of higher eukaryotes. A computational approach to predict the fold of DUF283 proposes that DUF283 could possibly adapt a dsRBD fold (Dlakic, 2006).

We have also investigated a role of PAZ and dsRBD domains in human Dicer. Mutations of four conserved amino acids in the Dicer PAZ domain were shown to have strong inhibitory effect on the dsRNA cleavage activity of Dicer. We proposed that PAZ domain is responsible for the recognition of a substrate end by binding the 2-nt 3' overhang. Structure of related PAZ domains from Argonaute proteins have revealed the similarity to the oligonucleotide binding fold and determined that the domain specifically recognizes the 2 nt 3' overhang of the duplex or the 3'-OH end of a single-stranded RNA (Lingel et al., 2004; Liu et al., 2004; Ma et al., 2004). By binding the overhang, the PAZ domain together with the connector helix and the platform DUF283 domain (see above) ensures

the appropriate distance between the end of the substrate and the active center of the enzyme.

We have determined that although human Dicer dsRBD sequence differs quite significantly from general dsRBD consensus, it is remarkably conserved among vertebrates and more conserved than any other Dicer domain (Fig. 1, Chapter 3). We have found that dsRBD of Dicer is able to bind 130-, 50- and 30-bp-long dsRNAs. However, the 19-bp siRNAs are bound with significantly lower affinity. This could reflect two different binding modes of Dicer. During the dsRNA/pre-miRNA cleavage dsRBD would be engaged with product recognition and binding. At the effector step, siRNA would be bound to Dicer by other domains (i.e. PAZ and RNase III), dsRBD would not contribute to the product binding, possibly lowering the affinity of the enzyme for its cleavage product and favoring siRNA/miRNA release or handing over to downstream components, such as Ago proteins (Fig. 5, Chapter 3). Recently RNA-binding properties of dsRBD-containing protein, RDE-4, were also investigated (Parker et al., 2006). It was found that purified RDE-4 binds with higher affinity to long dsRNA. This is in agreement with our observations on Dicer dsRBD and XLRBPA dsRBD2 and suggests that the better binding of longer substrates is an intrinsic property of the dsRBD. We have found that dsRBD of Dicer acts as an atypical Nuclear Localization Signal (NLS) (M. Doyle, unpublished results). To determine whether the NLS activity of dsRBD depends on RNA binding we have characterized 6 different mutants that were defective in dsRNA binding. The ability of the dsRBD

of human Dicer to localize to the nucleus appears to be RNA-independent (M. Doyle, unpublished data).

Function of the helicase domain of Dicer still remains unknown. The double stranded siRNAs must be unwound at a certain stage of the reaction to generate a single-stranded siRNA, which will base-pair to the mRNA targeted for degradation after being incorporated into RISC; the same is true for the initial, double-stranded form of miRNA resulting from the processing of pre-miRNA by Dicer. So far we could not demonstrate ATPase activity in Dicer preparations, suggesting that function of this domain is possibly regulated by additional factors. Also, it was shown previously that mutation of the conserved lysine residue in the nucleotide binding site (P-loop motif) has no impact on Dicer activity (Zhang et al., 2002). Dicer orthologs are present in almost all eukaryotic organisms, but the differences exist between from different organisms. In *C. elegans* and also in the case of *Drosophila* Dicer-1, the substrate cleavage seems to ATP-dependent. *Drosophila* Dicer-1 is also a component of the holo-RISC, whose formation is stimulated by ATP (Pham et al., 2004). Possibly, helicase domain of *Drosophila* Dicer-1 plays a cleavage-independent role in this process. *Giardia intestinalis* Dicer is devoid of the helicase domain, but it can complement the RNAi functions of *S. pombe* strain deleted of the endogenous Dicer despite that the latter contains the helicase domain. In *Dictyostelium discoideum*, the putative homolog of Dicer also lacks the ATPase/helicase domain, and a domain with a homology to the Dicer ATPase/helicase domain is present in the RNA-dependent-RNA-polymerase-like protein (Martens et al., 2002). It was proposed that the

helicase/ATPase domain of Dicer could promote translocation of the enzyme along the dsRNA, or a structural rearrangement of the substrate required for the cleavage. So far there is no evidence to support either of the proposed functions. It is possible that *C. elegans* and *Drosophila* Dicer-1 cleave dsRNA by a processive mechanism requiring ATP hydrolysis, while the mammalian Dicer might function in a distributive, ATP-independent way. However, other possibilities are also conceivable. For example, preparations of human Dicer might lack some of the components required for the ATPase activity that are present in worm and fruit fly extracts. This explanation would be consistent with the observed low catalytic efficiency of the mammalian enzyme. However, no ATP effect was observed when extracts or Dicer immunoprecipitates from mammalian cells were assayedfor dsRNA cleavage activity (Billy et al., 2001). Analysis of purified Dicer proteins and the complexes that the enzyme forms in cells from different systems like *C. elegans*, *Drosophila* and mammals might provide further information about the role of the ATPase/helicase domain and might help to understand organism-specific differences.

In an attempt to characterize Dicer containing complexes in human cells we have found that Dicer consistently copurifies with the TRBP protein. TRBP has previously been found to perform several functions. It is involved in inhibition of the interferon (IFN)-induced dsRNA regulated protein kinase PKR (Daher et al., 2001), modulation of HIV-1 gene expression through its association with TAR (Dorin et al., 2003) and control of cell growth (Benkirane et al., 1997; Lee et al., 2004a). A mouse TRBP homologue, Prbp, was shown to function as a

translational regulator during spermatogenesis, and Prbp knock-out mice were male sterile and usually died at the time of weaning (Zhong et al., 1999).

To validate the Dicer−TRBP interaction, we performed co-immunoprecipitation experiments using either extracts from human cells or purified proteins. We found that Dicer and TRBP directly interact and their association is RNA independent. Using stable, TRBP knock-down (TRBP-kd) cell lines we analyzed the role of TRBP in miRNA- and siRNA- mediated silencing. Using the RISC-mediated mRNA reporter cleavage we determined that TRBP is required for either the assembly or functioning of RISC. We have also found that the response to exogenous siRNA was significantly reduced in TRBPkd cells. We compared the pre-let7 processing activity of TRBPkd cell extracts with that of the Dicer-kd and the wild type cell extracts. However, despite that the extracts prepared from TRBPkd cells are less active in pre-miRNA processing, the steady state levels of several miRNAs did not significantly differ between knock down and control cells, and there was no apparent accumulation of pre-miRNAs in TRBP-kd cells. Taken together, our data suggest that TRBP is required for the assembly and function RISC in mammalian cells, and it may also stimulate the cleavage of pre-miRNAs by Dicer.

Two other reports also described the TRBP−Dicer partnership (Chendrimada et al., 2005; Lee et al., 2006). Two observations described in the Chendrimada et al paper do not agree with our results. First, they found that depletion of TRBP results in a decrease of steady-state levels of miRNAs, whereas in our analysis the miRNA content was not significantly changed.

Importantly, our finding were confirmed by Narry Kim's laboratory reporting that using similar TRBPkd cell line no significant changes in miRNA levels are detectable (Lee et al., 2006). Second, in contrast to our findings, Chendrimada et al reported that knockdown of TRBP destabilizes Dicer, and vice versa (Chendrimada et al., 2005). However, the authors did not analyze the levels of TRBP and Dicer in total extracts prepared from cells depleted in either protein but the analysis was performed with Ago-2 immunoprecipitates. Hence, it is likely that depletion of Dicer or TRBP affects the ability of the partner protein to form a complex with Ago-2 rather than causing the destabilization of proteins. In this context it is important to mention that the knockout of Prbp, the mouse homologue of TRBP, causes a relatively mild phenotype in mice (Zhong et al., 1999), in contrast to the Dicer knockout that is embryonic lethal (Bernstein et al., 2003). This argues against the possibility that depletion of TRBP destabilizes the Dicer protein.

In the follow-up research, the laboratory of Shiekhattar reports that human cells contain already preassembled complex containing Dicer, TRBP and Ago-2, capable of binding siRNA duplexes (Chendrimada et al., 2005) and that the complex containing only these three components in able to determine the asymmetry of the siRNA duplex and correctly incorporate the guide strand for mRNA cleavage (Gregory et al., 2005). In addition, the authors claim that the RISC activity does not require ATP hydrolysis since it is stimulated by addition of ATP and also non-hydrolyzable ATP-analogs or even inorganic phosphate. The findings of this work are however not entirely conclusive, because the

experiments were performed using cell extract immunoprecipitates, and it is not certain whether no other protein factors copurifying with the Dicer/Ago-2/TRBP complex. Requirements for additional factors could explain why reconstitution of active RISC using recombinant proteins was unsuccessful so far.

Our findings that mammalian Dicer forms a complex with a dsRBD protein TRBP confirm the notion that class II and class III RNase III enzymes such as Drosha and Dicer, generally require dsRBD protein partners for their function. *Drosophila* Loquacious and R2D2 are two *Drosophila* dsRBD proteins that associate with Dicer-1 and Dicer-2, acting in miRNA and siRNA pathways, respectively (Forstemann et al., 2005; Lee et al., 2004b; Liu et al., 2003; Pham et al., 2004; Saito et al., 2005). Loquacious and Dicer-1 are essential for efficient pre-miRNA processing, and also participate in gene silencing that is triggered by artificial dsRNA hairpins (Forstemann et al., 2005; Saito et al., 2005). R2D2−Dicer-2 complex is able to define the asymmetry siRNAs and ensure its proper loading into RISC (Tomari et al., 2004). Our observation that TRBP is required for efficient cleavage of pre-miRNA *in vitro* and for the function of RISC programmed with either endogenous miRNA or transfected siRNA into cells indicates that TRBP combines at least some functions that are performed separately by Loquacious and R2D2 in *Drosophila*. We investigated, using both cell extracts and recombinant proteins, whether Dicer and TRBP are involved in sensing the thermodynamic stability of the 5' ends of the siRNA strands in the same way as Dicer-2 and R2D2. These experiments, using 5-iodo-U-modified siRNAs, have not produced conclusive results (our unpublished results).

Another three-dsRBD protein, PACT, 42% identical to TRBP, is expressed in mammals. In contrast to TRBP, which inhibits PKR, PACT has a stimulatory effect on PKR. The effects of TRBP and PACT on PKR activity are mediated by the C-terminal dsRBDs, which have no detectable dsRNA-binding activity (Gupta et al., 2003). In addition to effects on PKR, the C-terminal domains of PACT and TRBP can mediate homodimerization of both proteins (Daher et al., 2001). C-terminal dsRBD of TRBP is also involved with association with Dicer, what raises the possibility that RNAi and PKR pathways could be connected and subjected to reciprocal regulation by protein−protein interactions. Moreover, PACT has been recently implicated in interaction with Dicer and the depletion of PACT has been shown to strongly affect the accumulation of mature miRNAs in cells (Lee et al., 2006). Both PACT and TRBP are known as regulators of PKR (Gupta et al., 2003). PKR is a dsRNA-dependent serine/threonine protein kinase, which phosphorylates eIF2alpha on Ser51 to cause a general reduction of protein synthesis (Dar et al., 2005; Dey et al., 2005). Besides eIF2alpha, PKR is known to phosphorylate several other proteins, such as NFAR-1, NFAR-2, and human protein phosphatase 2A (PP2A) regulatory subunit B56alpha (Saunders et al., 2001; Xu and Williams, 2000). It is conceivable that the components of RISC might be regulated by PKR through phosphorylation and that PACT and TRBP may regulate PKR activity to control the RISC activity. Alternatively, it is possible that there exists competition between the RNA silencing pathway and the PKR pathways, because the third dsRBD of both PACT and TRBP is responsible for their interaction with Dicer as well as for the regulation of PKR. It certainly would

be interesting to investigate the possibility of the crosstalk between the RNA silencing and the PKR pathways. As both RNAi and IFN–PKR pathways have a role in antiviral responses, communication between them could be envisioned. In the future, it would be interesting to find out how Dicer interaction with TRBP and PACT affect RNA silencing and other defence pathways in normal and virus infected cells. Recently it has been reported that the HIV-1 TAR-binding protein Tat functions as an RNAi suppressor, sequesters TRBP and thus compromises the activity of Dicer (Bennasser et al., 2005).

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SUPPLEMENTARY CHAPTER 1

ATP Modulates siRNA Interactions with an Endogenous Human Dicer Complex

ATP modulates siRNA interactions with an endogenous human Dicer complex

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ABSTRACT

Short interfering RNA (siRNA) binding by Dicer is important for RNA interference in *Drosophila*, but human Dicer (hDcr) has been reported to lack siRNA binding activity. We used native gel electrophoresis to characterize the siRNA-binding activity of endogenous hDcr-containing complexes in extracts from human cells. We identified a complex (D) that contains hDcr, as demonstrated by antibody supershift. Complex D appears to contain double-stranded siRNAs, and requires structural features of authentic siRNAs. Glycerol gradient sedimentation indicates that Complex D is ~250 kDa, slightly larger than hDcr alone. In addition, we found that purified recombinant hDcr (rhDcr) alone has siRNA binding activity. Complex D migrates more slowly than the rhDcr/siRNA complex in a native gel, suggesting that it contains at least one additional factor. hDcr directly contacts siRNAs within Complex D, as indicated by crosslinking. The endogenous complex is significantly enhanced by ATP, unlike the siRNA-binding activity of purified rhDcr, suggesting the existence of additional factors that can enforce the ATP dependence of endogenous hDcr/siRNA interactions. Complex D could impinge upon the RISC assembly pathway in humans, similar to an analogous complex in *Drosophila*.

Keywords: Dicer; RNA-induced silencing complex (RISC); RNA interference (RNAi); short interfering RNA (siRNA)

INTRODUCTION

RNA interference (RNAi) is a sequence-specific response to double-stranded RNA (dsRNA) that down-regulates genes at the level of mRNA stability (Fire et al. 1998). A class III ribonuclease, Dicer, cleaves long dsRNA into 21–23-nucleotide (nt) duplexes. These short interfering RNAs (siRNAs) are subsequently unwound and incorporated into RISC, where they direct the site-specific cleavage of complementary mRNA (for reviews, see Meister and Tuschl 2004; Filipowicz 2005; Sontheimer 2005; Tomari and Zamore 2005).

Although the RNAi response was discovered nearly a decade ago, the pathway's mechanism remains only partially characterized. Much of the initial characterization has been done in *Drosophila*, leading to the identification of numerous RISC components (for reviews, see Sontheimer 2005; Tomari and Zamore 2005). Dcr-2 is required to initiate RISC assembly on siRNAs (Lee et al. 2004; Pham et al. 2004). An additional protein, R2D2, is needed to stabilize Dcr-2 and to facilitate siRNA binding (Liu et al. 2003). The R2D2/Dcr-2/siRNA complex progresses to intermediate complexes that then assemble into holo-RISC (Pham et al. 2004; Tomari et al. 2004a).

Active RISC contains only one strand of the siRNA duplex, and strand selection is thought to occur in an asymmetric fashion. Studies suggest that the strand with the less thermodynamically stable 5′ end is favored for RISC assembly (Khvorova et al. 2003; Schwarz et al. 2003; Reynolds et al. 2004). Intriguingly, there is evidence to suggest that the R2D2/Dcr-2 heterodimer senses this asymmetry, with Dicer preferentially binding the 5′ end of the strand favored for RISC entry (the guide strand), and R2D2 preferentially binding the 5′ end of the other (passenger) strand (Tomari et al. 2004b).

In addition to the extensive body of work in *Drosophila*, there have also been important advances made in human RNAi biochemistry. A human RISC of ~160 kDa was partially purified (Martinez et al. 2002) and copurifies with the proteins Argonaute1 and Argonaute2 (Ago1 and Ago2). Human Ago2 has been shown to act as the endonuclease responsible for target cleavage (Liu et al. 2004; Meister et al. 2004; Song et al. 2004; Yuan et al. 2005). A functional human ortholog of R2D2 was only recently reported (Chendrimada et al. 2005; Haase et al. 2005).

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A single Dicer enzyme generates siRNAs and miRNAs in humans and other mammals, but conflicting data exist on whether mammalian Dicer participates in later stages of the RNAi pathway (Martinez et al. 2002; Doi et al. 2003; Chendrimada et al. 2005; Kanellopoulou et al. 2005; Kim et al. 2005; Rose et al. 2005; Siolas et al. 2005).

Despite our growing body of knowledge, much remains unknown about the mechanism of RNAi in higher eukaryotes. It was recently shown that hAgo2 can bind single-stranded siRNA (Liu et al. 2004; Rivas et al. 2005), but double-stranded siRNA-binding proteins in the human RISC assembly pathway are only beginning to be characterized (Chendrimada et al. 2005; Haase et al. 2005). Although hDcr binds long dsRNA (Provost et al. 2002; Zhang et al. 2002), two reports state that it does not possess double-stranded siRNA-binding activity (Provost et al. 2002; Chendrimada et al. 2005). This raises questions about the similarity of mammalian RNAi to the *Drosophila* system, in which siRNA binding by R2D2/Dcr-2 is required to initiate RISC assembly (Liu et al. 2003; Lee et al. 2004; Pham et al. 2004; Tomari et al. 2004a). In addition, the role of hDcr's ATPase domain remains unknown, since purified rhDcr's only biochemical activities reported to date (dsRNA substrate binding and processing, and pre-miRNA processing) are ATP-independent (Provost et al. 2002; Zhang et al. 2002). To address these and other issues, we set out to identify and characterize human siRNA-binding complexes. We identified one such complex in which hDcr interacts directly with the siRNA in an ATP-dependent fashion.

RESULTS AND DISCUSSION

siRNP complexes in human cell extracts

We used native gel electrophoresis to identify and characterize siRNA-containing complexes in human cell extracts and to determine whether hDcr was present in any of these complexes. After incubating HEK 293 S100 extract with a radiolabeled siRNA duplex, we observed several complexes on a native gel (Fig. 1A, lane 1). These complexes formed in HEK 293 and HeLa S100 extracts, both of which are competent for in vitro RNAi (data not shown). To assess the siRNA-binding specificity of the complexes, we altered the structure of the nucleic acid substrate. Only one of the complexes (denoted by an arrow in Fig. 1) was significantly reduced when the guide strand was modified with a 5′-terminal 2′-deoxy-5′-methoxythymidine residue, which blocks 5′ phosphorylation (Fig. 1A, lane 3). The same complex was unaffected when the guide strand contained a 5′-terminal 2′-deoxythymidine residue (Fig. 1A, lane 2). Because a 5′-phosphate group is important for siRNA function (Nykänen et al. 2001), this result suggests that the 5′-methoxy-sensitive complex alone has the binding specificity expected of a bona fide RNAi complex, and we characterized this complex further.

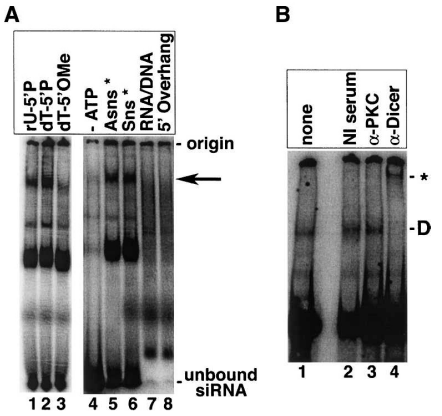

FIGURE 1. An siRNA-binding activity from human cell extracts requires ATP and contains hDcr. (*A*) Radiolabeled siRNA was incubated with HEK 293 S100 extract and analyzed by native gel electrophoresis. The samples shown in lanes *1–3* and *5–8* contained ATP and an ATP-regenerating system. The sample in lane *4* was depleted for ATP. The siRNAs in lanes *1–3* were labeled at the 3′ end of the antisense (guide) strand; the passenger strand contained a 5′ hydroxyl. In lanes *4–8*, the siRNAs were labeled at their 5′ end with [γ^{32}P]-ATP, and the unlabeled strand contained a 5′ phosphate. In lanes *4* and *5*, the guide strand was labeled, and in lanes *6–8* the passenger strand was labeled. "rU-P" denotes the unmodified siRNA with a uridine 5′-phosphate at its 5′ terminus. "dT-P" denotes an siRNA modified with a 2′-deoxythymidine 5′-phosphate at its 5′ terminus. "dT-OMe" denotes a 2′-deoxy-5′-methoxythymidine-modified siRNA. The asterisk (*) in lanes *5* and *6* denotes the labeled strand. "RNA/DNA" refers to an siRNA duplex in which the guide strand is DNA rather than RNA. "5′ Overhang" indicates a duplex with 2-nt 5′ overhangs. The 5′-methoxy-sensitive complex is denoted by an arrow. (*B*) Radiolabeled siRNA was incubated with extract and α-hDcr antibodies, and then analyzed by native gel electrophoresis. As negative controls, nonimmune serum ("NI serum") and a nonspecific antibody ("α-PKC") were also used. Lane *1* ("none") indicates the mobility of the hDcr-containing species (Complex D) in the absence of any antibody. The supershifted complex is denoted by an asterisk (*).

The 5′-phosphate-dependent siRNA binding activity is greatly enhanced by ATP, because ATP depletion severely reduced complex levels (Fig. 1A, lane 4). The complex appeared equally when either strand of a reportedly asymmetric siRNA (Schwarz et al. 2003) was labeled, suggesting that it contains double-stranded RNA (Fig. 1A, cf. lanes 5 and 6). Moreover, no single-stranded RNA was observed in partially purified fractions containing this complex (data not shown). It did not form on a hybrid RNA/DNA duplex (Fig. 1A, lane 7) or on an siRNA containing a 2-nt 5′ overhang as opposed to the characteristic 2-nt 3′ overhang (Fig. 1A, lane 8). Furthermore, its formation is sequence-independent, as it formed on multiple, unrelated siRNAs (data not shown). The complex formed very early in time-course experiments (data not shown). Target cleavage activity did not cofractionate with the complex in a 10%–30% glycerol gradient, indicating that it is not a mature form of RISC.

The 5′-methoxy-sensitive complex contains hDcr

Some of the features of the 5′-methoxy-sensitive complex are reminiscent of an siRNA- and Dcr-2-containing complex (R1) that initiates RISC assembly in *Drosophila* (Pham et al. 2004). To address whether hDcr is present within the complex, we performed antibody supershift assays using our native gel system. In the presence of nonimmune serum or an unrelated antibody, the complex formed as usual (Fig. 1B, lanes 1–3). In contrast, incubation with antibodies raised against hDcr (Billy et al. 2001) resulted in the disappearance of the complex and the concomitant appearance of a more slowly migrating species (Fig. 1B, lane 4). We conclude that the complex contains the hDcr protein, and we now refer to it as Complex D.

To further characterize Complex D, we set out to partially purify it. We incubated HEK 293 S100 extract with radiolabeled siRNA and subjected the reaction to 10%–30% glycerol gradient sedimentation. Fractions were analyzed by native gel, yielding a clear Complex D-containing peak (Fig. 2A). To confirm that this complex was truly the D complex, we repeated the antibody supershift assay. In the presence of the α-hDcr antibody, the partially purified complex shifted in a native gel (Fig. 2B, cf. lanes 1 and 4). This shift did not occur when the complex was incubated with nonimmune serum or a nonspecific antibody (Fig. 2B, lanes 1–3). Therefore, the siRNA-bound species observed in the glycerol gradient peak corresponds to hDcr-containing Complex D. We estimated the size of Complex D as 250–300 kDa based on comparison with the sedimentation behavior of the U1 snRNP, which has a known molecular weight of 240 kDa (Stark et al. 2001).

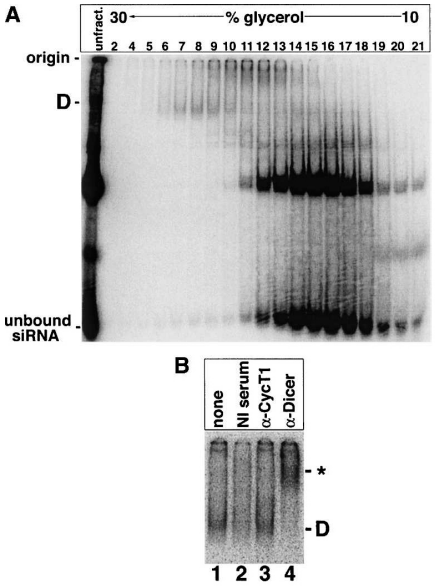

FIGURE 2. Partially purified D complex contains hDcr. (*A*) HEK 293 S100 extract was incubated with radiolabeled siRNA and sedimented on a 10%–30% glycerol gradient. Fractions were collected from the bottom of the gradient and analyzed by native gel electrophoresis. Complex D peaked in fractions 6–10. (*B*) As in Figure 1B, except that the Complex D-containing fraction from *A* was substituted for S100 extract. Antibodies against cyclin T1 ("α-CycT1") were used as a negative control.

hDcr directly contacts the siRNA within Complex D

Since hDcr and the siRNA coexist within the D complex, we tested whether the siRNA directly contacts the hDcr protein by crosslinking with a radiolabeled siRNA containing a photo-activatable 5-iodouracil at its 3′ end. This siRNA was incubated with extract, exposed to 312-nm light, and fractionated by 10%–30% glycerol gradient sedimentation. We analyzed fractions by native gel and SDS-PAGE, and observed a radiolabeled protein of ~220 kDa that cofractionated with Complex D (Fig. 3A). This protein was specifically immunoprecipitated with an α-hDcr antibody both from irradiated crude extract and from irradiated Complex D-containing gradient fractions (Fig. 3B), indicating that it is hDcr. Interestingly, hDcr was photo-crosslinked with equal or slightly greater intensity when the guide strand contained the 5-iodouracil at its 3′ end, as opposed to when the passenger strand contained the 5-iodouracil at its 3′ end (Fig. 3C). This is contrary to results obtained in *Drosophila* (Tomari et al. 2004b), where Dcr-2 crosslinks much more efficiently to the 3′ end of the passenger strand. This suggests that siRNA asymmetry might not be sensed identically in humans and *Drosophila*.

Recombinant human Dicer binds siRNA

Purified recombinant hDcr (~220 kDa) sediments more slowly than endogenous Dicer from mouse extracts (Zhang et al. 2004), suggesting that most hDicer stably associates with one or more additional proteins (probably TRBP; Chendrimada et al. 2005; Haase et al. 2005). Our estimation that Complex D is ~250–300 kDa (see above) is consistent with this. To further test the possibility that the D complex contains additional proteins besides hDcr, we compared its electrophoretic mobility to that of siRNA-bound purified recombinant Dicer (rhDcr) in native gels. As shown in Figure 4A, we were able to detect rhDcr binding to the radiolabeled siRNA, contrary to earlier reports (Provost et al. 2002; Chendrimada et al. 2005). The siRNA-binding activity of rhDcr was ATP-independent (Fig. 4B), in contrast to the siRNA-binding activity of endogenous hDcr within Complex D (Fig. 1A). This rhDcr/siRNA complex migrated faster than Complex D on a native gel (Fig. 4C), consistent with the possibility that the endogenous complex contains one or more factors in addition to hDcr.

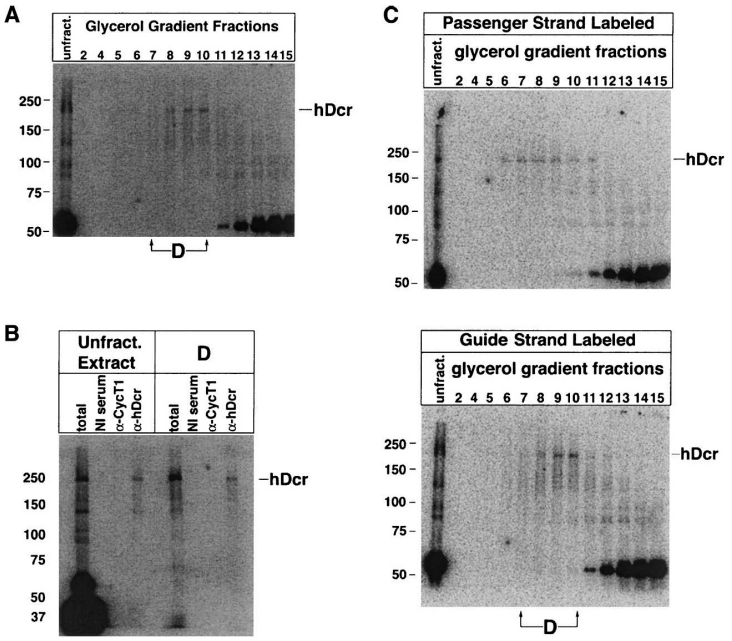

FIGURE 3. hDcr directly contacts the siRNA within Complex D. (*A*) Labeled siRNA containing a 5-iodouracil at position 20 was incubated in extract and irradiated with 312-nm light. The crosslinked mixture was sedimented on a 10%–30% glycerol gradient, and fractions were separated on a 6% polyacrylamide-SDS gel. (*B*) Crosslinked extract or Complex D-containing fractions from *A* were added to Protein-A sepharose beads bound with nonimmune serum ("NI serum") or antibodies against cyclin T1 or hDcr. The immunoprecipitated samples were denatured and analyzed by electrophoresis on a 6% polyacrylamide-SDS gel. (*C*) Radiolabeled siRNA duplex containing a 5-iodouracil on either the guide or passenger strand was incubated with extract, crosslinked with 312-nm light, and sedimented on a 10%–30% glycerol gradient. Fractions were collected and analyzed by electrophoresis in 6% polyacrylamide-SDS gels. The labeled strand (containing the 5-iodouracil) is indicated at the *top* of each panel, and the Complex D-containing fractions are indicated *below*. The mobility of hDcr is given on the *right*.

Is Dicer involved in RISC assembly in mammals?

A significant question is whether Complex D is a true functional intermediate in RISC assembly in humans. This would usually be assessed by preforming the complex and challenging it with a large excess of competitor substrate, and then testing its ability to progress into a mature, functional complex upon the addition of other required components. However, despite exhaustive attempts, we have been unable to conduct such a "chase" experiment with Complex D, because control experiments indicate that the siRNA off-rate is too high to allow the complex to survive the challenge with a large amount of competitor siRNA.

Several published reports are consistent with roles for Dicer in RISC assembly in humans. Following dsRNA processing by rhDcr, the enzyme remains associated with some of the siRNA product, and product inhibition could account for rhDcr's low catalytic efficiency under multiple turnover conditions (Zhang et al. 2002). Furthermore, HeLa cells depleted of either hDcr or its recently identified binding partner TRBP show a decreased RNAi response, even when triggered by siRNAs (Doi et al. 2003; Chendrimada et al. 2005; Haase et al. 2005). Three recent reports demonstrated that hDcr substrates are more efficient than siRNAs at triggering RISC activity in human cells (Kim et al. 2005; Rose et al. 2005; Siolas et al. 2005), consistent with the possibility that Dicer processing potentiates RISC assembly. Finally, Dcr-2 plays a role in RISC assembly in *Drosophila* (Lee et al. 2004; Pham et al. 2004; Tomari et al. 2004a), and a lack of involvement for hDcr in human RISC assembly would indicate a dramatic and unexpected difference between the otherwise well conserved RNAi pathways in these two organisms.

Although numerous reports suggest that Dicer may be involved in mammalian RISC assembly, several lines of evidence indicate that it is not strictly required. In contrast to in vivo results in human cells (Doi et al. 2003; Chendrimada et al. 2005; Haase et al. 2005), HeLa extract immuno-depleted of hDcr retained full siRNA-triggered RISC activity (Martinez et al. 2002). Additionally, Dicer-null mouse embryonic stem cells were capable of siRNA-mediated RISC activity (Kanellopoulou et al. 2005). Although these data indicate that RISC is capable of assembling in the absence of hDcr, they do not exclude the possibility that hDcr normally plays a role in enhancing RISC assembly and function.

In addition to identifying a Dicer/siRNA complex in human cell extract, we have presented evidence that purified rhDcr alone is capable of binding to siRNA. This binding activity is contrary to previous reports (Provost et al. 2002; Chendrimada et al. 2005). The rhDcr/siRNA complex migrates faster on a native gel than Complex D, suggesting that the endogenous complex contains one or more additional factors. It is likely that an additional factor is TRBP (Chendrimada et al. 2005; Haase et al. 2005), although TRBP is not absolutely required for Dicer/siRNA binding, as appears to be the case for Dicer-2 and R2D2 in *Drosophila*. It is possible that Complex D can function as a precursor in RISC assembly, analogous to a similar complex observed in *Drosophila*.

Most Dicers (including hDcr and *Drosophila* Dcr-2) contain an apparent N-terminal ATPase domain (Carmell and Hannon 2004), but the function of this domain has been enigmatic. Missense mutations in the Dcr-2 ATPase do-

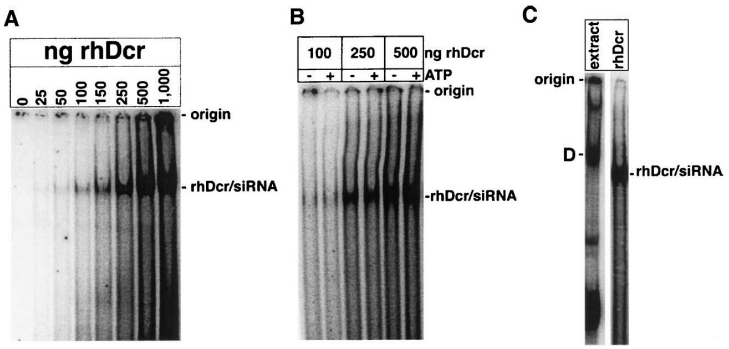

FIGURE 4. Recombinant hDcr binds siRNA. (*A*) Increasing amounts of rhDcr protein were incubated with radiolabeled siRNA and analyzed by native gel electrophoresis. The amount of rhDcr is given at the *top* of each lane. (*B*) As in *A*, except that 0.5 mM ATP and an ATP-regenerating system were either omitted or included, as indicated at the *top* of each lane. (*C*) Radiolabeled siRNA was incubated with either HEK 293 S100 extract or 250 ng rhDcr and analyzed on a native gel.

main block dsRNA processing activity but have no effect on subsequent phases of RNAi (Lee et al. 2004). Furthermore, ATP is required for efficient dsRNA cleavage by *Drosophila* extract (Bernstein et al. 2001; Nykänen et al. 2001) and by recombinant Dcr-2 (Liu et al. 2003). In contrast, ATP depletion (Provost et al. 2002; Zhang et al. 2002) or a mutation predicted to block ATP binding (Zhang et al. 2002) has no effect on dsRNA processing by rhDcr. Thus, ATP appears to be required for dsRNA processing in insects but not mammals. In the case of siRNA binding activity, the situation is reversed: *Drosophila* Dcr-2/R2D2 does not require ATP for siRNA association (Liu et al. 2003; Pham et al. 2004), whereas endogenous hDcr/siRNA interaction within Complex D does require ATP (Fig. 1A). Although the D complex's ATP requirement cannot yet be ascribed to ATP binding or hydrolysis by the hDcr N-terminal domain, these results suggest that this domain is likely to play different roles in different Dicer enzymes. Interestingly, the siRNA binding activity of purified rhDcr (Fig. 4B) indicates that hDcr/siRNA interactions are not intrinsically ATP-dependent, and suggests that additional factors present in human cell lysates may enforce Complex D's ATP dependence. Uncovering the basis for this effect will illuminate the roles of Dicer and ATP in human RNAi.

MATERIALS AND METHODS

General methods

HEK 293 and HeLa S100 extracts were prepared as previously described (Dignam et al. 1983) except the final dialysis buffer contained 10% glycerol. The *Pp*-luc siRNA sequences used in this study were previously described in Nykänen et al. (2001). The siRNA was labeled either at the 3′ end of the antisense strand with an [α^{32}P]-cordycepin 5′-triphosphate (Perkin Elmer, 5000 Ci/mmol) using poly(A) polymerase (Amersham), or on the 5′ end of either strand with [γ^{32}P]-ATP (MP Biomedicals, 7000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs) according to the manufacturers' instructions. Duplex siRNAs were annealed as described in Pham et al. (2004).

For all experiments outlined in this study, RNAi reactions contained 75% extract (vol/vol), 3 mM MgOAc, 2.5 mM DTT, 0.5 mM ATP, 12.5 mM creatine phosphate, 0.015 μg/mL creatine kinase (Calbiochem), 4.5 units RNAguard (Amersham), and 5000–10,000 cpm siRNA duplex. The reactions were incubated at 37°C for 1 h, unless otherwise specified. Standard RNAi reactions were 10 μL. For ATP depletion experiments, ATP, creatine phosphate, and creatine kinase were omitted and replaced with 10 mM glucose and 0.05 U/mL hexokinase (Calbiochem). The ATP depletion reactions were incubated at 37°C for 15 min before addition of siRNA. In the rhDcr experiments, the extract was removed and replaced with 1 μL purified rhDcr-HisC (Zhang et al. 2002) and 6.5 μL lysis buffer. For the rhDcr experiments lacking ATP, creatine phosphate, creatine kinase, and ATP were omitted from the reaction and replaced with water. RISC activity was assayed as described in Pham et al. (2004).

Native gel electrophoresis

Reactions to be run on a native gel were supplemented with 10% (vol/vol) glycerol before incubation at 37°C. The native gel system was as described in Pham et al. (2004). No heparin was added to the reactions. For the antibody supershift assay, RNAi reactions (or reactions with partially purified Complex D in place of extract) were incubated at 37°C for 30 min, after which nonimmune serum or antibodies against protein kinase C (PKC), cyclin T1 (CycT1), or hDcr (D347, as described in Billy et al. 2001) were added. The mixtures were incubated at 4°C for 5 h, then analyzed by native gel electrophoresis.

Glycerol gradient sedimentation

An RNAi reaction (400 μL, 400,000 cpm) was layered on a 10%–30% glycerol gradient in Buffer A (Zhang et al. 2004). The gradient was centrifuged at 36,000 rpm for 24 h at 4°C in a Beckman SW 41 Ti rotor. Fractions (~550 μL) were collected by dripping from the bottom of the tube.

Photo-crosslinking

Pp-luc siRNA containing a 5-iodouracil at position 20 of one strand was 5′-end-labeled and annealed as described above. This siRNA was used in an RNAi reaction, then placed on an inverted 96-well plate in ice, covered with a Petri dish lid to minimize exposure to shortwave UV light, and irradiated with a 312-nm lamp (15 W, 1000 μW/cm^2) for 10 min. For the immunoprecipitations, Protein A-sepharose beads (24 mg) were rinsed with a wash buffer (20 mM

Tris-HCl pH 7.5, 200 mM NaCl, 0.5% Igepal, 2.5 mM $MgCl_2$, Protease Inhibitors-EDTA [Roche]), blocked in a 500 μL mixture of BSA, glycogen, and carrier tRNA (10 μg/mL each in wash buffer) for 15 min at 4°C, and washed three times. Nonimmune human serum or antibodies against either CycT1 or hDcr were added and the beads were gently rocked at room temperature for 1 h, then washed 3 × 500 μL with wash buffer. A crosslinking reaction or Complex D-containing fraction from a crosslinked gradient were added to the beads with 300 μL wash buffer, gently rocked for 4 h at 4°C, and washed 4 × 200 μL with wash buffer. The samples were eluted with 1× SDS-PAGE sample buffer, heated at 100°C for 10 min, then loaded onto a 6% SDS-PAGE gel.

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SUPPLEMENTARY CHAPTER 2

The Chromatoid Body of Male Germ Cells: Similarity with Processing Bodies and Presence of Dicer and microRNA Pathway Components

The chromatoid body of male germ cells: Similarity with processing bodies and presence of Dicer and microRNA pathway components

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The chromatoid body is a perinuclear, cytoplasmic cloud-like structure in male germ cells whose function has remained elusive. Here we show that the chromatoid body is related to the RNA-processing body of somatic cells. Dicer and components of microRNP complexes (including Ago proteins and microRNAs) are highly concentrated in chromatoid bodies. Furthermore, we show that Dicer interacts with a germ cell-specific chromatoid body component, the RNA helicase MVH (mouse VASA homolog). Thus, chromatoid bodies seem to operate as intracellular nerve centers of the microRNA pathway. Our findings underscore the importance of posttranscriptional gene regulation and of the microRNA pathway in the control of postmeiotic male germ cell differentiation.

Argonaute | microRNA | spermatogenesis | RNA processing

The complex and highly elaborated differentiation program of male germ cells involves multiple and finely tuned levels of gene control (1). During late steps of spermiogenesis there is a striking cessation of transcription, occuring in concert with drastic epigenetic modifications that result in chromatin compaction (2). Concomitantly there are extensive posttranscriptional storing and processing of mRNAs (3).

RNA interference (RNAi) and microRNA (miRNA) pathways are evolutionarily conserved control mechanisms that use RNA molecules to inhibit gene expression at the level of mRNA degradation, translational repression, or chromatin modification and silencing (4–6). RNAi has been shown to be present throughout spermatogenesis in mice (7), but its function and cellular control during germ cell development remain uncertain.

Two classes of 21- to 25-nt small RNAs, small interfering RNAs (siRNAs) and miRNAs, act as sequence-specific regulators of gene expression (5). siRNAs mediate degradation of mRNAs having sequences fully complementary to their sequence, whereas miRNAs are proposed to regulate gene expression by inhibiting protein synthesis through imperfect base-pairing to the 3′ UTR of target mRNAs (4, 5). Both siRNA and miRNA precursors are processed to mature small RNAs in the cytoplasm of cells by the large endonuclease Dicer (4, 8). Mature miRNAs and siRNAs are assembled into miRISC and siRISC (miRNA- and siRNA-induced silencing complexes, respectively), which subsequently act on their targets by translational repression or mRNA cleavage. Essential components of RISC complexes are the members of the Argonaute family of proteins (9). These proteins share the so-called PAZ and PIWI domains and are classified into two subfamilies depending on sequence similarity to either *Arabidopsis Argonaute1* or *Drosophila Piwi* (9, 10). In mammals, Argonaute1 subfamily members, Ago1 to Ago4, have been shown to be involved in the RNAi/miRNA pathway (11, 12). All four members of the PIWI subfamily are expressed mainly in testis (10), and two of them, MIWI and MILI, are crucial for progression through spermatogenesis in mouse (13, 14).

In many organisms, including *Drosophila*, germ cells are characterized by the accumulation of dense fibrous material into a cytoplasmic structure, called germplasm or nuage (15). The chromatoid body is suggested to be a mammalian counterpart of nuage on the basis of its structural features and protein composition (16). It is a finely filamentous, lobulated perinuclear granule located in the cytoplasm of mammalian postmeiotic round spermatids. Both *Drosophila* nuage and mouse chromatoid body contain an ATP-dependent DEAD-box RNA helicase, VASA [in the mouse MVH (mouse VASA homolog)] (17–19). *Drosophila* nuage is thought to function as a site for translational regulation of many important mRNAs, the VASA protein seemingly playing a central role in this process (20). The role of the chromatoid body in the mouse is still elusive, although it was proposed to be involved in RNA storing and processing. In addition to MVH, some other proteins have been reported to localize in the chromatoid body, most of which are known to be involved in RNA metabolism (16). Although there is no DNA in the chromatoid body (21), the presence of RNA has been suggested (22–25).

Here we demonstrate the physical relationship between the chromatoid body and the miRNA machinery in haploid germ cells. Dicer and components of miRISC (also referred to as microRNP), such as various members of the Argonaute family and several miRNA species, concentrate in the chromatoid body. The presence of Dcp1a and other specific proteins in the chromatoid body provides evidence of a functional analogy with the processing bodies (P-bodies), cytoplasmic structures found in somatic cells (ref. 26 and references therein). We have also found that Dicer interacts with the VASA homolog MVH. These findings shed new light on the function of the chromatoid body, a structure whose existence has been known for decades (16) but whose role has remained unknown. In addition, our results underscore the importance of the miRNA pathway in the control of postmeiotic male germ cell differentiation.

Results

Colocalization of the VASA Homolog MVH and MIWI in Chromatoid Body. The highly restricted localization of MVH in the chromatoid body has been used as specific marker for this structure (18). We confirmed this observation by performing immunostaining

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Abbreviations: siRNA, small interfering RNA; miRNA, microRNA; RNAi, RNA interference; RISC, RNA-induced silencing complex; MVH, mouse VASA homolog; P-body, processing body; DIG, digoxigenin.

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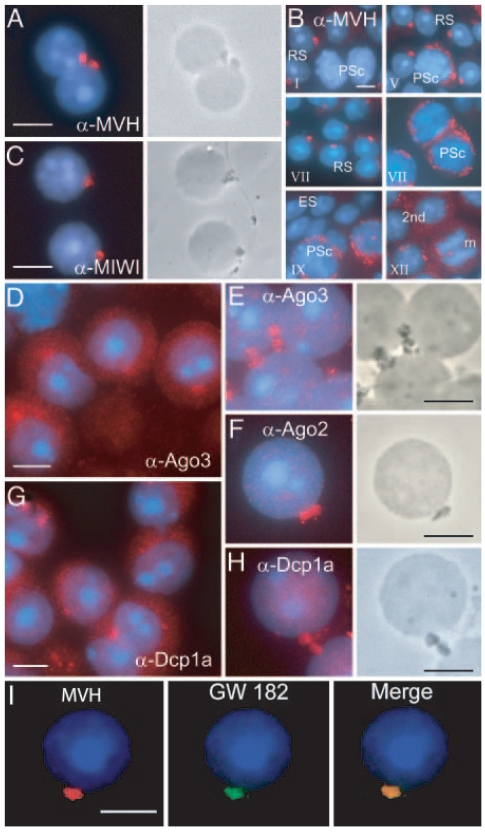

Fig. 1. Localization of MVH and Ago proteins in chromatoid body. (*A*) Drying-down slides containing germ cells from stages II–V were labeled with anti-MVH antibody (red). The localization in chromatoid body was confirmed by parallel phase contrast microscopy. (*B*) Expression of MVH in chromatoid bodies during spermatogenesis. Squash preparations from stages I, V, VII, IX, and XII were stained with anti-MVH antibody (red) and studied by fluorescence microscopy. RS, round spermatid; PSc, pachytene spermatocyte; 2nd, secondary spermatocyte; m, meiotic division. (*C*) Localization of MIWI in chromatoid body. Germ cells from stages II–V were labeled with anti-MIWI antibody. (*D* and *E*) Ago3 is concentrated in chromatoid bodies. Germ cell squash preparations (*D*) at stage IV or drying-down slides containing germ cells from stages II–V (*E*) were immunolabeled by polyclonal anti-Ago3 antibody (red). (*F*) Ago2 was also shown to be concentrated in chromatoid bodies by using polyclonal anti-Ago2 antibody. (*G* and *H*) Localization of the P-body marker, the decapping enzyme Dcp1a, in chromatoid bodies. Germ cell squash preparations at stages III–IV (*G*) or drying-down slides from stages II–V (*H*) were immunostained by polyclonal anti-Dcp1a antibody. (*I*) Chromatoid body localization of a GW body marker protein. Patient sera against human GW182 protein (18033; a gift from Marvin J. Fritzler, University of Calgary, Calgary, Canada) were used for localization studies. For MVH detection, rabbit polyclonal anti-MVH antibody was used as a primary antibody. Alexa Fluor 594 anti-rabbit IgG or Alexa Fluor 488 anti-human IgG were used as secondary antibodies, and DAPI was used to stain nuclei (blue). (Scale bars: 5 μm.)

with anti-MVH antibodies on male germ cells isolated from stages II–V of seminiferous epithelial cycle (Fig. 1*A*). Chromatoid bodies are clearly visible in parallel phase contrast microscopy as electron-dense areas (Fig. 1*A*). Using the drying-down technique to prepare the cell slides, the cytoplasm is partially lost to expose the chromatoid body, which usually stays in contact with the nucleus.

We analyzed the formation of the chromatoid body along the differentiation of postmeiotic germ cells. Stage-specific sequential squash preparations show MVH expression in chromatoid bodies throughout the development of round spermatids (Fig. 1*B*). Expression is detected in the cytoplasm of pachytene spermatocytes at stages IV–V within filamentous–granular structures. In round spermatids, the chromatoid body becomes finely compacted, and usually there is only one per cell. The RNA-binding protein MIWI has been shown to have a granular distribution in the cytoplasm of haploid round spermatids (14), but it was unclear whether these granules corresponded to chromatoid bodies. We demonstrate that MIWI is indeed concentrated in chromatoid bodies, colocalizing with MVH (Fig. 1*C*).

Argonaute Proteins and P-body Components in the Chromatoid Body. The presence of MIWI in chromatoid bodies prompted us to analyze the localization of Ago subfamily members to establish a possible relationship with the miRNA pathway. The function of Ago proteins in male germ cells is presently unknown. Squash preparations at stage IV showed a granular cytoplasmic localization of Ago3 (Fig. 1*D*). Although Ago3 was diffused throughout the cytoplasm, the signal was concentrated on one cytoplasmic spot in close contact with the nucleus (Fig. 1*D*). This granule corresponds to the chromatoid body, as revealed by immunofluorescence of germ cell slides and parallel phase contrast (Fig. 1*E*). Ago2 was also concentrated in the chromatoid body (Fig. 1*F*). It has been recently shown that in somatic cells Ago proteins localize in cytoplasmic P-bodies (27–29). We therefore analyzed the localization of a P-body marker, the decapping enzyme component Dcp1a (30), in male germ cells. Strikingly, Dcp1a was also concentrated in chromatoid bodies (Fig. 1 *G* and *H*). The distribution of Dcp1a along spermatogenesis is dynamic. Although it has a granular cytoplasmic localization in both meiotic and postmeiotic germ cells, in late pachytene spermatocytes there seem to be several Dcp1a granules (data not shown), which then concentrate postmeiotically in the chromatoid body (Fig. 1*H*). Two other P-body markers, the 5′-to-3′ exonuclease Xrn1 (data not shown) and the RNA-binding protein GW182 (Fig. 1*I*) (31), were also found in chromatoid bodies.

Because P-body components localize to the chromatoid body, we questioned whether the male germ cell-specific MVH could potentially be targeted to P-bodies in somatic cells. To this end, FLAG-tagged MVH and HA-Ago2 or HA-Ago3 were ectopically expressed in HeLa cells. Indeed, MVH colocalized to the same cytoplasmic granules with Ago2 and Ago3 (Fig. 6, which is published as supporting information on the PNAS web site). MVH mutants containing only the N terminus [FLAG-MVH(1–199)] or lacking the central ATPase domain (FLAG-MVHΔ283–503) (see Fig. 5) failed to colocalize with Ago proteins (Fig. 6). MVH-positive granules were shown to correspond to P-bodies by coimmunostaining of endogenous Dcp1a (Fig. 6). Thus, the P-bodies of somatic cells and the chromatoid body of male germ cells share a number of significant similarities.

miRNAs Are Concentrated in Chromatoid Bodies. We performed *in situ* hybridizations using probes specific for various miRNAs known to be expressed in testis to study whether their localization overlaps with Ago proteins in chromatoid bodies. The analysis on squash preparation of stages V–VI, or drying-down slides of germ cells from stages II–VI, revealed high concentration of miR-21 and let-7a in perinuclear granules in round spermatids (Fig. 2 *A–D*). The signal overlapped with MVH signal, clearly demonstrating that miRNAs accumulated in chromatoid bodies. miR-122a also colocalized with MVH in chromatoid body (Fig. 2*E*). A mutant let-7a probe, used as a negative control, gave no signal (Fig. 2*F*). Interestingly, no enrichment of signal was detected with the prelet7a RNA probe, indicating that

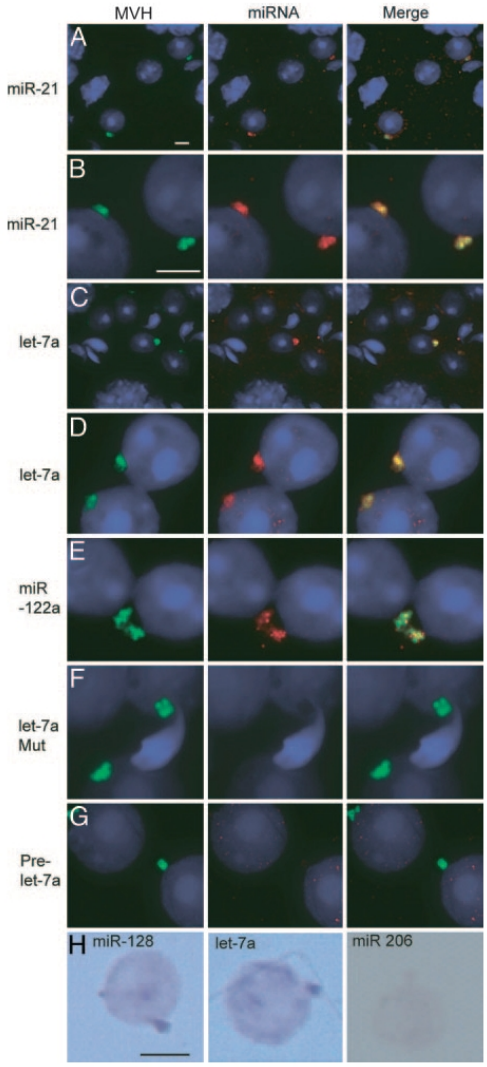

Fig. 2. Localization of miRNA in chromatoid bodies. (*A–G*) *In situ* hybridization was performed with DIG-labeled locked nucleotide probes specific for different miRNAs. Except in *A* and *C*, samples fixed by the drying-down method were used for analysis. In *A* and *C*, squashed samples from stage II–VI seminiferous tubules were used. The miRNA-specific probes used in each panel are indicated. The prelet-7a probe was designed to detect the prelet-7a but not the mature let-7a. let-7a Mut is a mutant version of let-7a-specific probe. After *in situ* hybridization, miRNA signals were detected with the Roche fluorescent antibody enhancer set for DIG detection (red). MVH signals are in green, and the nucleus was stained with DAPI (blue). The merged pictures are also shown. (*H*) detection of miRNA by the colorimetric method. miR-206-specific probe is used as a negative control. (Scale bars: 5 μm.)

it is primarily the processed form of let7a that localizes to chromatoid bodies (Fig. 2*G*). Chromatoid body localization of miR-128 and let-7a, but not of nontestis-expressed control miRNA miR-206, was also demonstrated by *in situ* hybridization of drying-down slides by using the colorimetric detection method (Fig. 2*H*).

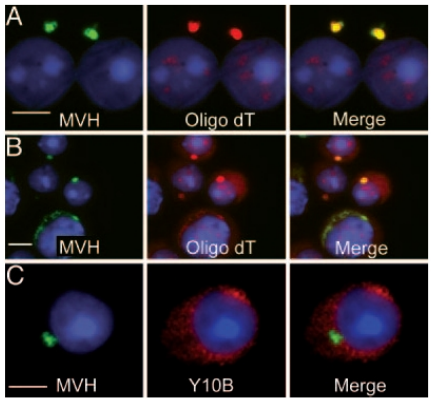

Fig. 3. Poly(A)$^+$ RNA is concentrated in chromatoid bodies. A Cy3-labeled oligo(dT) probe was used to detect mRNAs with a polyA tail in cells fixed by the drying-down method (*A*) and also in cells of stage V–VII squash preparations (*B*). After *in situ* hybridization with Cy3-labeled oligo(dT), MVH signals were revealed by immunofluorescence (green). DAPI was used to stain the nucleus. (*C*) Ribosomal RNA localization in round spermatids. Ribosomes localized to cytoplasmic regions (red) distinct from the chromatoid body positive for MVH (green). (Scale bars: 5 μm.)

Localization of the RISC components and miRNAs in chromatoid bodies motivated us to study whether mRNA molecules, the natural targets for miRNA-mediated regulation, also concentrate in chromatoid bodies. *In situ* hybridization using oligo(dT) probe clearly demonstrated accumulation of mRNAs in chromatoid bodies (Fig. 3 *A* and *B*). Interestingly, antibodies specifically recognizing rRNA did not stain chromatoid body (Fig. 3*C*); likewise, antibodies recognizing the small ribosomal subunit protein S6 showed no signal overlapping with the MVH-positive chromatoid bodies (data not shown), indicating that active translation at this specific stage of spermatogenesis occurs in locations distinct from chromatoid bodies.

Dicer Is Concentrated in the Chromatoid Body. The presence of Ago proteins and miRNAs in the chromatoid body directed us to study Dicer expression during spermatogenesis. Dicer is present in both meiotic spermatocytes and postmeiotic round spermatids but not in elongated spermatids (Fig. 4*A*). Expression is constant along the stages of the seminiferous epithelial cycle (Fig. 4*A*). We have also established that the Dicer protein in testis is enzymatically active (Fig. 4*B*). Immunostaining of germ cell slides from specific stages revealed that the expression of Dicer is indeed concentrated in chromatoid bodies (Fig. 4*C*). Finally, a detailed analysis along the differentiation of male germ cells shows a stage-dependent distribution of Dicer in the chromatoid body: In step 1 round spermatids, Dicer expression is still very low, but it increases at stages VI–VII. At stage VIII, Dicer levels in the chromatoid body decrease significantly. Thus, Dicer localization to the chromatoid body is highly dynamic, being at its peak coordinately with the other elements of the miRNA machinery.

Dicer Interacts with the VASA Homolog MVH. Although Dicer is known to interact with other proteins involved in RNA silencing (refs. 32 and 33 and references therein), its presence in the chromatoid body raised the possibility that it could interact with germ cell-specific elements, in particular MVH. FLAG-tagged MVH and EGFP-tagged Dicer were ectopically coexpressed in COS-1 cells and then coimmunoprecipitated (Fig. 5*A*). We found that MVH and Dicer interact. We have used a number of

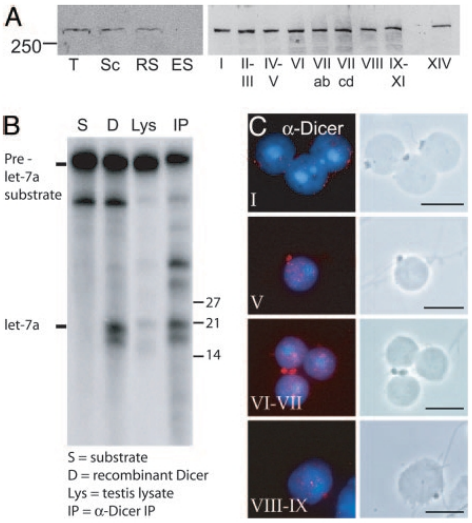

Fig. 4. Localization of Dicer in chromatoid body. (*A*) Expression of Dicer protein in testis. Western blot analysis of the protein extracts of rat total testis cells (T), pachytene spermatocytes (Sc), round spermatids (RS), and elongated spermatids (ES), as well as of different stages of the rat seminiferous epithelial cycle (indicated as Roman numerals I–XIV), was performed by using anti-Dicer antibody. (*B*) premiRNA-processing activity in testis. prelet-7a substrate was processed when testis extract (Lys) or anti-Dicer immunoprecipitate (IP) was added to the reaction. Reaction with recombinant Dicer (D) was used as a positive control. (*C*) Dicer is expressed in the chromatoid body of late round spermatids. The drying-down slides were immunostained with the anti-Dicer 349 antibody and studied by both fluorescence microscopy (red, Dicer; blue, DNA) and phase contrast microscopy to confirm the chromatoid body localization. Similar staining was obtained with anti-Dicer 350 antibody. (Scale bars: 10 μm.)

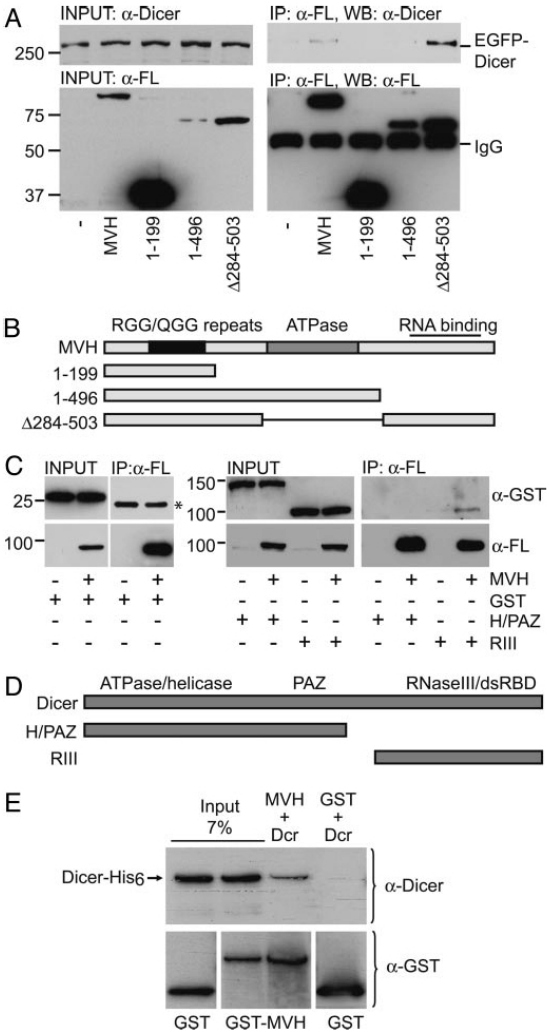

Fig. 5. Dicer interacts with MVH. (*A*) Interaction of the full-length EGFP-Dicer with MVH. IP was performed from COS-1 lysates overexpressing EGFP-Dicer and FLAG-MVH, FLAG-MVH(1–199), FLAG-MVH(1–496), or FLAG-MVHΔ284–503 with anti-FLAG antibody (α-FL), and the immunoblotting was performed by using either anti-Dicer antibody to detect coimmunoprecipitated Dicer or anti-FLAG antibody to detect MVH. (*B*) Schematic diagram of the MVH constructs used in this study. (*C*) The RNaseIII region of Dicer mediates the interaction with MVH. GST, GST-Helicase/PAZ containing the N-terminal helicase domain and the central PAZ domain of Dicer (H/PAZ), or GST-RNaseIII containing the C-terminal RNaseIII region and dsRNA binding domain of Dicer (RIII) was coexpressed with FLAG-MVH. After immunoprecipitation with anti-FLAG antibody, immunoblotting was done with anti-GST or anti-FLAG antibody. An asterisk indicates the IgG light chain. (*D*) Schematic representation of the Dicer mutants used in this study. (*E*) MVH and Dicer interact *in vitro*. Recombinant GST-MVH and Dicer-6xHis were incubated with glutathione Sepharose. After binding and washes, beads were run into a polyacrylamide gel, and immunoblotting was performed with anti-GST or anti-Dicer antibody.

MVH deletions to establish the protein domain involved in Dicer interaction (Fig. 5 *A* and *B*). The mutants MVH(1–199) and MVH(1–496) did not interact with Dicer, despite containing the central ATPase motif and the N-terminal RGG/QGG repeats known to be involved in protein/RNA interactions (17). This result shows that the C terminus of MVH, which contains a RNA-binding domain (17), is required for Dicer interaction (Fig. 5*A*). Interestingly, the MVHΔ284–503 deletion, which lacks the central ATPase motif, interacts with Dicer more efficiently than the full-length MVH protein. This finding may suggest a negative regulatory role of this region in controlling the interaction with Dicer.

Dicer is a multidomain protein that includes a RNA helicase/ATPase domain, the DUF283 and PAZ signatures, two neighboring RNase III-like domains, and a dsRNA binding domain (34). Our coimmunoprecipitation experiments demonstrate that the C-terminal RNaseIII region is sufficient for interaction with MVH (Fig. 5*C*). In support of this observation, a large portion of the Dicer protein containing the N-terminal ATPase/helicase domains and the central PAZ signatures did not interact with MVH (Fig. 5 *C* and *D*). Interestingly, HIWI, the human homolog of mouse MIWI, has also been shown to interact with the C terminus of Dicer (32). This interaction was used as a positive control (data not shown). Finally, the direct interaction between MVH and Dicer was confirmed by *in vitro* interaction assays by using bacterially generated recombinant GST-MVH and Dicer-6xHis (Fig. 5*E*). Thus, MVH appears to be a male germ cell-specific component of the Dicer complex.

Discussion

The molecular nature and function of the chromatoid body in germ cells have been elusive and debated for many years (16).

Our findings support a scenario where the chromatoid body would function as an RNA storing and processing structure. Specifically, the presence of Dicer/Argonaute and miRNAs reveals that the chromatoid body occupies a privileged position in posttranscriptional control of gene expression through the small RNAs pathway.

To date, very little was known about the function of siRNA and miRNA pathways and the expression and localization of the components of these pathways during spermatogenesis. RNAi has been shown to be active during the whole spermatogenesis program (7), and many miRNAs whose expression is enriched in testis have been identified (35, 36). Here we demonstrate that the main component of the RNA-silencing pathway, Dicer, is expressed in both meiotic and postmeiotic cells at all stages of the seminiferous epithelial cycle.

Dicer processes miRNA precursor molecules folded into dsRNA-like hairpins to mature miRNAs. These are subsequently transferred to the miRISC complex, where the effector phase of the process takes place (4, 8). A direct interaction between Dicer and Ago proteins may be required for the transfer of miRNAs to the effector complex (8). The presence in the chromatoid body of several miRNAs, of the enzyme that processes them, and of proteins essential for the effector phase of mRNA silencing strongly supports the notion that functional miRNA-mediated mRNA control is taking place in this compartment. Interestingly, a potential connection between germplasm (nuage) and miRNA pathway has also been suggested in *Drosophila*. Indeed, mutation of the nuage protein Maelstrom causes mislocalization of two miRNA pathway proteins, Dicer and Ago2 (37).

MIWI, a member of the PIWI subfamily of Argonaute proteins, localizes to the chromatoid body (Fig. 1). Argonaute1 subfamily members are widely expressed in many tissues. In contrast, all four members of PIWI subfamily are expressed mainly in testis (10), but their role in the RNAi pathway is still obscure (9). MIWI associates with ribohomopolymers, and specifically with transcripts of CREM-target genes, suggesting a functional connection of MIWI to RNA processing (13). Importantly, mice deficient for either MIWI or CREM display a similar block in spermatogenesis (13, 38). Because MIWI and the Argonaute1 subfamily members Ago2 and Ago3 colocalize in the chromatoid body (Fig. 1), it is tempting to speculate that MIWI may operate as a male germ cell-specific miRNA pathway component involved in functional mRNA regulation in testis.

In yeast and mammalian somatic cells, mRNAs targeted to translational repression and degradation accumulate in cytoplasmic P-bodies, where the enzymes involved in the RNA decay pathway are also concentrated (30, 39–41). Recently, Ago proteins, and also miRNAs and miRNA-repressed mRNAs, were demonstrated to localize in P-bodies in mammalian cells (27–29), suggesting that RNA silencing and RNA decay pathways may take place in the same cellular compartments. The current model proposes that mammalian miRNAs inhibit the initiation of translation of mRNAs that subsequently accumulate in P-bodies for storage (29). We have demonstrated that several P-body markers are highly concentrated in chromatoid bodies (Fig. 1). These results suggest that the chromatoid body of male germ cells and P-bodies in somatic cells are functionally related, both acting as a site for mRNA decay and mRNA translational repression by the miRNA pathway. Thus, our findings provide an attractive interpretation of the phenomenon of translational repression that occurs postmeiotically in spermatids (42, 43).

One significant result reported here is that an essential component of the chromatoid body, the RNA helicase/ATPase MVH, interacts with Dicer. MVH may operate as an anchoring element for RNA-silencing components, or it might have a more functional role. Interestingly, the turnover of the RISC cleavage activity depends on ATP, suggesting that release of the cleaved mRNA halves may involve RNA helicases (8, 44). Thus, our finding reveals MVH as a potential germ cell-specific candidate for providing helicase/ATPase activity to the RISC complex.

The chromatoid body is endowed with the remarkable property of moving very actively and three-dimensionally in the cytoplasm of round spermatids. During these movements chromatoid bodies make frequent contacts with the nuclear envelope. Continuity in electron-dense material between the nucleus and the chromatoid body through nuclear pore complexes has been observed (16). One hypothesis suggests that rapidly moving chromatoid bodies collect mRNA getting out from the nucleus. Interestingly, miRNA precursors processed in the nucleus are exported to the cytoplasm through nuclear pore complexes (45). Based on our findings we suggest a model in which premiRNAs transported to the cytoplasm are loaded through nuclear pores to the chromatoid body. Thus, the chromatoid body functions as a subcellular concentration site for components of the miRNA pathway, centralizing the miRNA posttranscriptional control system in the cytoplasm of haploid male germ cells.

Materials and Methods

Immunofluorescence. Squash preparations or drying-down slides (46) were fixed in 4% paraformaldehyde, and immunofluorescence was performed by using specific polyclonal or monoclonal antibodies and Alexa Fluor 594 and 488 secondary antibodies (Molecular Probes).

miRNA *in Situ*. Locked nucleotide probes were labeled with digoxigenin (DIG) by using the terminal transferase 3′ DIG-tailing kit (Roche). Fixed cells were permeabilized in 70% ethanol, rehydrated in 2× SSC for 15 min, and hybridized overnight at 37°C with 15 ng of the probe. After three washes, slides were incubated with anti-DIG alkaline phosphate-conjugated Fab fragment (1:5,000; Roche), and signals were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in the dark. In colocalization studies the Fluorescent Antibody Enhancer Set for DIG Detection (Roche) was used.

RNA Processing Assays. The internally ^{32}P-labeled prelet-7 RNA was dissolved in water and renaturated by incubation at 90°C for 1 min. Processing assays (50 μl) were carried out as previously described in refs. 34 and 47. ^{32}P-labeled substrate (3–5 fmol) was incubated with a testis extract or anti-Dicer immunoprecipitate in buffer containing 20 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 75 mM NaCl, and 10% glycerol at 37°C. RNA was extracted with phenol/chloroform and analyzed by 10% denaturing PAGE.

Immunoprecipitation. COS-1 cells transfected with indicated plasmids were collected and lysed in a buffer containing 50 mM Tris·HCl (pH 8.0), 170 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, and 1:1,000 protease inhibitor mixture. Immunoprecipitation was performed with anti-FLAG M2 (Sigma) monoclonal antibody and protein G Sepharose (Amersham Pharmacia Biosciences). After washes, proteins were released in 2× Laemmli sample buffer. Immunoblotting was performed by anti-GST (1:1,000), anti-FLAG M2 (1:1,000), or anti-Dicer 349 (1:1,000) antibody.

Recombinant Proteins and *in Vitro* Interaction. Recombinant GST-MVH was purified from the BL21 *Escherichia coli* strain with glutathione Sepharose (Amersham Pharmacia) and eluted from the beads with 20 mM glutathione. Recombinant Dicer was purified as described (47). *In vitro* protein interactions with recombinant proteins were analyzed in buffer containing 30 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, 100 mM NaCl, and 0.5% Nonidet P-40 by using standard methods.

Specific reagents and detailed additional procedures are fully

described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

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