The molecular architecture of human Dicer

Pick-Wei Lau1,2, Keelan Z Guiley2, Nabanita De2, Clinton S Potter1,3, Bridget Carragher1,3 & Ian J MacRae2

Dicer is a multidomain enzyme that generates small RNAs for gene silencing in eukaryotes. Current understanding of Dicer structure is restricted to simple forms of the enzyme, whereas that of the large and complex Dicer in metazoans is unknown. Here we describe a new domain localization strategy developed to determine the structure of human Dicer by EM. A rearrangement of the nucleosome core, compared to the archetypal Giardia lamblia Dicer, explains how metazoan Dicers generate products that are 21–23 nucleotides in length. The helicase domains form a clamp-like structure adjacent to the RNase III active site, facilitating recognition of pre-miRNA loops or translocation on long dsRNAs. Drosophila melanogaster Dicer-2 shows similar features, revealing that the three-dimensional architecture is conserved. These results illuminate the structural basis for small RNA production in eukaryotes and provide a versatile new tool for determining structures of large molecular machines.

Small regulatory RNAs, such as microRNAs (miRNAs) and short interfering RNAs (siRNAs), are involved in a myriad of biological processes ranging from viral defense to brain development. The RNase III enzyme Dicer performs a fundamental role in small RNA biogenesis by cleaving double-stranded RNA (dsRNA) substrates into functional small RNAs of a discrete size, typically 21–23 nucleotides (nt). Small RNA products of Dicer are incorporated into large multiprotein complexes termed RNA-induced silencing complexes (RISC). RISC and RISC-like complexes use the small RNAs as guides for the sequence-specific silencing of cognate genes through mRNA degradation and translational repression and heterochromatin formation.

In living cells, Dicer enzymes often function within larger protein complexes required for initiation of RNA silencing pathways. In Tetrahymena thermophila, Dcr2 is physically coupled to the RNA-dependent RNA polymerase Rdr1 for the biogenesis of siRNAs. In D. melanogaster, Dcr-2 interacts with the protein R2D2 and facilitates siRNA loading into Ago2 (ref. 8). In Caenorhabditis elegans, Dcr-1 associates with more than 20 other protein factors and exists in at least two different functional complexes that are distinctly responsible for initiating the endogenous and exogenous arms of the RNA interference (RNAi) pathway. In addition to generating small RNA duplexes, Dicer itself functions as a molecular scaffold in all of these complexes.

Central to the activity of Dicer is its ability to recognize dsRNA, to generate precisely sized products and, in some cases, to translocate along a long substrate. Structural and mechanistic insights into these activities have proved difficult, because the metazoan Dicers are large and complicated proteins, recalcitrant to crystallization. Previous studies have primarily focused on simple forms of the enzyme, either Dicers from lower eukaryotes or isolated domains from higher eukaryotic Dicers. Consequently, although the structures of many of the individual domain components of Dicer have been established (Fig. 1a), the overall architecture of the enzyme remains unknown.

To date, two EM reconstructions of human Dicer have been reported. Both describe an L-shaped particle composed of several morphologically discrete regions (Fig. 1b), and a working model for the domain architecture of Dicer has emerged. However, this model has not been examined rigorously and seemingly contradicts several observed biochemical properties of the enzyme. The difficulty in testing the model stems from technical challenges associated with localizing individual domains in EM maps; this is particularly difficult in relatively small, asymmetric particles such as Dicer, which is less than 250 kDa. To overcome this issue, we devised a versatile, site-specific tagging strategy compatible with single-particle analysis, allowing us to establish the first experimentally validated structure of this essential enzyme. Our structure differs substantially from the model proposed previously, but is well aligned with the known biochemical properties of Dicer.

RESULTS

RNA recognition by PAZ occurs in the head of Dicer

Dicer generates small RNAs by cleaving dsRNAs ~22 base pairs from their open helical ends. Recognition of the dsRNA end is directly mediated by an RNA-binding domain called PAZ. We identified the position of the PAZ domain in the EM map of human Dicer by inserting the 15–amino acid AviTag sequence, a substrate for biotin-protein ligase, into a surface loop in the PAZ domain (between residues Lys916 and Glu917) and, after subsequent biotinylation, tagging with a monovalent form of streptavidin. Tagged proteins were purified and visualized by negative stain EM. We observed extra density, with the size and shape of streptavidin extending from the head of the L, in many unsupervised 2D class averages (Fig. 2a and

1National Resource for Automated Molecular Microscopy, The Scripps Research Institute, La Jolla, California, USA. 2Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, USA. 3Department of Cell Biology, The Scripps Research Institute, La Jolla, California, USA. Correspondence should be addressed to B.C. (bcarr@scripps.edu) and I.J.M. (macrae@scripps.edu).

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Supplementary Fig. 1). Eight independent 3D reconstructions of the tagged Dicer were generated by the random conical tilt (RCT) method, and the L-shaped portion of each reconstruction was aligned with the refined Dicer structure. The point of streptavidin attachment (estimated as the central point in the bridging region between Dicer and streptavidin densities) was then mapped onto the refined structure. The estimated attachment points lie within a 10-Å radius located at the front of the head region (Fig. 2b), revealing that the PAZ domain—and thus the site of dsRNA end recognition—is in the very top of the molecule, in the front of Dicer’s head.

**Platform domain is tightly associated with PAZ domain**

Upon recognition of dsRNA by PAZ, Dicer then cleaves the substrate ~22 nt from the open helical end. In the simple Dicer enzyme from the protozoan *G. lamblia*, a ‘platform’ domain separates PAZ from the RNase III catalytic site by a distance of ~70 Å, thereby providing the structural basis for production of RNAs 25–27 nt in length. Human Dicer has been proposed to use a similar measuring mechanism, although its products are 4 nt shorter. We identified the position of the platform domain in the human Dicer EM map by inserting the AviTag between residues Asp886 and Ser887. For platform-labeled particles, streptavidin density extended from the back of the head in 2D class averages (Fig. 2c and Supplementary Fig. 1), and 3D reconstructions from eight class averages mapped the point of attachment to a region of radius 10 Å in the back of the Dicer head (Fig. 2d). The position of the platform suggests that both PAZ and the platform are tightly associated, as in the case of *G. lamblia* Dicer.

**dsRNA cleavage by RNase III domains occurs in body**

It has previously been proposed that a fixed spacing between the PAZ and RNase III domains in human Dicer could lead to cleavage of dsRNA ~22 nt from the open helical end. To test this model directly, we tagged the RNase IIIb domain with streptavidin (residues Asn1780–Glu1800 were replaced with the AviTag). Streptavidin density was apparent in 2D class averages and extended from the body of the L, approximately 55 Å from the PAZ domain (Fig. 2e and Supplementary Fig. 1). The tagged RNase IIIb loop appears to be more mobile than the loops labeled in the PAZ and platform domains, as the estimated points of streptavidin attachment for eight different RCT models lie within a 20-Å radius, with the labeled loop extending out from the right side of the body (Fig. 2f). Consistent with this arrangement, a reconstruction of Dicer lacking the C-terminal double-stranded RNA-binding domain, which lies adjacent to the tagged loop in the RNase IIIb domain, is missing density from the right side of the body (Supplementary Fig. 2).

**Human Dicer core rearranged relative to G. lamblia Dicer**

Based on the positions of the streptavidin tags, we docked the platform–PAZ module and RNase III domains from the *G. lamblia* Dicer crystal structure into the EM map of human Dicer (Fig. 2g). Although the head easily accommodated the platform–PAZ module, the RNase III domains of *G. lamblia* Dicer could not be fit into the body of the EM map without a major rearrangement relative to the platform (Fig. 3). Rearranging these domains established a 3D model for the architecture of the human Dicer nuclease core. Conceptually, the core of the human enzyme is similar to *G. lamblia* Dicer; both have PAZ and RNase III domains separated by a specific distance. However, relative to their PAZ domains, the RNase III active sites of human and *G. lamblia* Dicer...
are offset from each other by a rotation of roughly 120° around the long axis of the human enzyme. Moreover, in human Dicer, the platform domain does not lie directly between PAZ and RNase III. Instead, a structurally undefined ‘ruler domain’ physically separates the two functional domains (Fig. 3a and Supplementary Fig. 3). These large-scale differences in 3D architecture probably reflect the fact that small RNAs in humans are 4 nt (one-third of a dsRNA helical turn) shorter than in G. lamblia; the human enzyme must attack a completely different face of its dsRNA substrates relative to their helical ends.

**Helicase forms clamp-shaped structure at base of Dicer**

In addition to differences in product length, human Dicer further differs from G. lamblia in the complexity of its accessory domains. Human Dicer contains an N-terminal helicase, which itself is composed of three predicted globular domains: HEL1, HEL2i and HEL2 (Fig. 1a). After comparing 2D class averages of full-length and helicase-deleted Dicer proteins, others have proposed that the helicase resides within the arm, or ‘baselimb’ of the L (ref. 17). However, the volume of the arm is too small to accommodate all three globular domains of the Dicer helicase. Indeed, the crystal structure of DDX3X, which was used previously as a model for the Dicer helicase17, is composed of only two globular domains and lacks any structure analogous to the HEL2i domain that is observed in the Dicer primary sequence. To investigate this issue, we generated 3D reconstructions of a truncated Dicer in which the three helicase domains (residues 1–604) were deleted (Fig. 4a). RCT reconstruction of Δhelicase-Dicer produced an oblong structure with dimensions similar to the head and body portion of Dicer. Similarly, a variety of projection-matching refinements of Δhelicase particles consistently produced structures resembling the head and body (Supplementary Fig. 4). We conclude that the deleted helicase forms not just the arm but the entire base of full-length Dicer.

Sequence homology suggests the Dicer helicase belongs to the RIG-I family of RNA helicases33. Indeed, the core helicase domains of RIG-I form a ‘C’ shape similar to the base of the Dicer L (Supplementary Fig. 5)34. To establish the orientation of the helicase domains, we determined the structure of a truncated Dicer protein with only the HEL1 domain (residues 1–211) deleted (Fig. 4a). The HEL1 reconstruction lacks density in the bottom corner of the L, corresponding to a mass of approximately 25 kDa, in agreement with the mass of the deleted HEL1 domain (Fig. 4b).

Based on the position of the HEL1 domain, we docked the crystal structure of duck RIG-I helicase34 into the base of the L (Fig. 4c). Notably, the RIG-I helicase and Δhelicase-Dicer reconstruction together accounted for the total density of the full-length Dicer map. The interior channel of the helicase, which constitutes the dsRNA binding site in RIG-I, is aligned with the central channel that runs up the body of the L (Supplementary Fig. 6). Thus, a single continuous channel runs through the clamp of the helicase, past the RNase III active site, and ends with the RNA-binding pocket of the PAZ domain (Supplementary Fig. 6). We propose that this channel is the major surface used by the enzyme for processing dsRNA.
Dicer architecture is conserved

The function of the Dicer helicase has been enigmatic: it has been suggested to contribute to substrate binding in human Dicer, facilitate pre-miRNA recognition in D. melanogaster Dcr-1, and catalyze translocation on long dsRNA substrates in C. elegans Dcr-1 and D. melanogaster Dcr-2 (refs. 23, 25). Considering the divergent functions reported for the Dicer helicase, we wondered how the architecture of Dicer varies between different species. To explore this issue, we extended our EM analysis to a sample of D. melanogaster Dcr-2. Dcr-2 is one of the best studied Dicer enzymes and differs from human Dicer in that it requires ATP to cleave dsRNA and is believed to couple ATP hydrolysis to translocation on long dsRNA. The 2D class averages of Dcr-2 contained many L-shaped particles similar to those observed in the class averages of human Dicer (Fig. 6a). Furthermore, projection-matching using Dcr-2 particles led to an L-shaped reconstruction with dimensions markedly similar to those of the human enzyme (Fig. 6b). We therefore conclude that despite clear functional differences among various forms of the enzyme, the overall three-dimensional architecture of Dicer is well conserved.

DISCUSSION

The structural analysis of Dicer presented here allows reconciliation of the seemingly disparate functions of the helicase observed in different Dicer homologs. Adjacent to the RNase III domains, the helicase is positioned to bind the stem-loops of pre-miRNAs (Fig. 7a, b). This explains how the helicase contributes to pre-miRNA binding in human Dicer and selective processing of pre-miRNAs in D. melanogaster Dcr-1 (ref. 24). For processive Dicers, the helicase could use ATP hydrolysis to translocate dsRNA into the nuclease core of the enzyme. The helicase is positioned to remain bound to long dsRNAs after cleavage and formation of each siRNA product, providing a structural basis for processivity on long substrates (Fig. 7c).

The previously proposed structural model for Dicer suggested the opposite orientation for the nuclease core, with the PAZ domain in the body, adjacent to the helicase, and RNase III domains in the head. This model implies that the ends of dsRNA substrates bind within the body and extend out past the head, never directly interacting with the helicase. It has thus been difficult to explain how the helicase could facilitate processivity or substrate binding if it is positioned on the opposite side of the molecule from where the dsRNA feeds in. We have now experimentally determined the 3D positions of the PAZ, RNase III and helicase domains, allowing us to exclude this model and...
cohesively integrate a structural view of the enzyme with its known biochemical functions. Moreover, Dicer enzymes typically function as central components in larger complexes that are required for initiation of diverse RNA silencing pathways\(^\text{3-10}\). Therefore, establishing the correct structure of Dicer is necessary for understanding the mechanisms of the multiprotein assemblies needed for a wide range of gene silencing processes.

**METHODS**

Methods and any associated references are available in the online version of the paper at [http://www.nature.com/nsmb/](http://www.nature.com/nsmb/).

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**AUTHOR CONTRIBUTIONS**

C.S.P., B.C. and I.J.M. conceived of the project. P.-W.L., K.Z.G. and N.D. prepared the samples. P.-W.L. carried out the data collection. P.-W.L., C.S.P., B.C. and I.J.M. analyzed the data. P.-W.L. and I.J.M. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Generation of AviTag Dicer constructs and truncated Dicer constructs. DNA encoding the AviTag sequence (LNDILEAQKIEWHEG) was cloned into specific positions corresponding to surface loops in a cDNA clone of human Dicer (NM_030621) in the plasmid pFastBac HT A (Invitrogen) within the PAZ (between residues Lys916 and Glu917), Platform (between residues Asp886 and Ser887) and RNase IIIb domains (replacing residues Asn1780–Glu1800).

Deletion mutants of human Dicer were amplified by PCR using the full-length cDNA clone as a template. The ΔHeli1 Dicer lacks amino acids 1 – 211, while the ΔHelicase constructs lacks amino acids 1 – 604. The cloning details are described in the Supplementary Methods.

Dicer expression and purification. All the Dicer constructs were produced and purified from Sf9 cells using the Bac-to-Bac system (Invitrogen), as described. Recombinant proteins were purified using a His6 tag, which was cleaved by treatment with TEV protease. The samples were then passed through a 5-ml HisTrap FF column, and the flow-through fraction was collected. Samples were then concentrated and analyzed by size-exclusion chromatography. Further details can be found in the Supplementary Methods.

Biotinylation and streptavidin labeling of AviTag Dicer. Partially purified AviTag Dicers were biotinylated after the His-Trap FF step (see above). The flow-through fraction from the His-Trap column was concentrated and exchanged into a buffer containing 250 mM potassium glutamate with 25 mM Tris, pH 8.0. Biotin ligase (BirA) is inhibited at high concentrations of sodium chloride. Biotinylation reactions were carried out in a reaction volume of 0.5 to 1 ml. One tenth the volume of a 1× buffer containing 100 mM ATP, 100 mM magnesium acetate and 500 µM n-biotin, pH 8, was added to the sample together with the purified BirA enzyme (20 µl of 5 mg ml−1). Biotinylation reactions were incubated for 1 h at 37 °C.

Following the biotinylation reaction, the samples were dialyzed extensively (against a low imidazole buffer) to remove excess biotin. The biotinylated Dicers were then bound to purified monovalent streptavidin. Biotinylation efficiency was typically about 60%. The streptavidin-tagged Dicer samples were purified from untagged proteins by applying the sample to a 1 ml HisTrap FF column. As only the streptavidin contained a His6 tag, non-biotinylated Dicers did not bind the column. The retained samples were eluted by increasing imidazole concentration and then concentrated and passed through a HiLoad 16/60 Superdex 200 column (GE Healthcare) to separate the Dicer–streptavidin complex from free streptavidin.

Negative staining and electron microscopy. Samples were adhered to C-flat grids coated with a layer of thin carbon. Heavy metal uranyl solution was used to embed and fix the sample. All data were acquired using a Tecnai F20 Twin transmission electron microscope operating at 120 keV, using a dose of ~20 e− Å−2 or a nominal defocus range of ~1 to ~3 µm. Images were automatically collected during multiple sessions at a nominal magnification of 50,000x or 62,000x at specimen-level pixel sizes of 0.151 nm or 0.131 nm, respectively. Images were recorded using either a Tietz F415 4 × 4 K pixel CCD camera (15 µm pixel) or Gatan 4 × 4 K pixel CCD camera, using LegoIno. Random conical tilt (RCT) experiments were carried out using the RCT node of Legoino, with image pairs taken at 0 and (−) 50 degrees. Additional details are available in the Supplementary Methods.

Image processing and model reconstructions. Image processing and model reconstructions were carried out using the Appion package. Particles were extracted from the raw micrographs using a reference-free method. For alignments and classifications, a combination of Xmipp and Spider protocols were used. The 3D reconstruction was done using a combination of SPIDER and EMAN reconstruction packages. Additional details are available in the Supplementary Methods.

Model fitting. All model fitting was done using Chimera. Map segmentations were carried out using Segger, available in Chimera. Additional details on modeling are available in the Supplementary Methods.

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