Structural and biochemical insights into 2'-O-methylation at the 3'-terminal nucleotide of RNA by Hen1

Chio Mui Chan^{a,1}, Chun Zhou^{a,1}, Joseph S. Brunzelle^b, and Raven H. Huang^{a,c,2}

^aDepartment of Biochemistry and ^cCenter for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801; and ^bLife Science Collaborative Access Team, Argonne National Laboratory, Argonne, IL 60439

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Small RNAs of ≈ 20 –30 nt have diverse and important biological roles in eukaryotic organisms. After being generated by Dicer or Piwi proteins, all small RNAs in plants and a subset of small RNAs in animals are further modified at their 3'-terminal nucleotides via 2'-O-methylation, carried out by the *S*-adenosylmethionine-dependent methyltransferase (MTase) Hen1. Methylation at the 3' terminus is vital for biological functions of these small RNAs. Here, we report four crystal structures of the MTase domain of a bacterial homolog of Hen1 from *Clostridium thermocellum* and *Anabaena variabilis*, which are enzymatically indistinguishable from the eukaryotic Hen1 in their ability to methylate small single-stranded RNAs. The structures reveal that, in addition to the core fold of the MTase domain shared by other RNA and DNA MTases, the MTase domain of Hen1 possesses a motif and a domain that are highly conserved and are unique to Hen1. The unique motif and domain are likely to be involved in RNA substrate recognition and catalysis. The structures allowed us to construct a docking model of an RNA substrate bound to the MTase domain of bacterial Hen1, which is likely similar to that of the eukaryotic counterpart. The model, supported by mutational studies, provides insight into RNA substrate specificity and catalytic mechanism of Hen1.

RNA interference | RNA methylation | RNA repair | X-ray crystallography

The last decade has witnessed the exciting discovery of small RNAs of 20–30 nt that regulate gene expression in eukaryotic organisms (1). To date, four classes of small RNAs, broadly defined as siRNAs, micro RNAs (miRNAs), Piwi interacting RNAs (piRNAs), and endogenous small interfering RNAs (esiRNAs), have been characterized. These small RNAs are incorporated into members of the Argonaute (Ago or Piwi) family proteins and, together with a set of associated proteins, form the RNA-induced silencing complex (RISC) (2–4). The small RNA in the RISC guides the complex to a target RNA through Watson–Crick base-pairing between the small RNA and the target RNA, resulting in the regulation of cellular function through the interaction of RISC with the target RNA. Based on our current knowledge, these small RNAs are involved in almost every aspect of biological process of eukaryotic organisms, including antiviral defense, cell proliferation, cell death, cell differentiation, developmental timing, transposon suppression, and stem cell maintenance.

Generation and maturation of these small RNAs usually involves two steps. First, long RNA precursors are cleaved by an endoribonuclease to produce small ≈ 20 –30 nt RNAs. The ribonuclease responsible for the cleavage is either the Dicer (for the generation of siRNAs, miRNAs, and esiRNAs) or Piwi (for the generation of piRNAs) protein. Second, all of the small RNAs in plants, and a subset of small RNAs in animals, are further modified through 2'-O-methylation at their 3'-terminal nucleotides, carried out by Hen1, a class of *S*-adenosylmethionine (AdoMet)-dependent RNA methyltransferase (MTase) (5–8). In plants, the modification is essential for the biological function of small RNAs as failure to carry out the modification

results in rapid degradation of small RNAs via polyuridylation at their 3'-unmodified ends (9, 10). The biological importance of small RNA methylation in animals is also emerging. For example, in *Tetrahymena thermophila*, loss of Hen1 results in defects in programmed DNA elimination by small RNAs and inefficient production of sexual progeny (11).

Although the amino acid sequences and architectures of Dicer and Argonaute proteins are conserved across the eukaryotic organisms possessing RNAi, Hen1 in plants is very different from its ortholog in animals, with the exception of the conservation of the MTase domain (Fig. S1). Hen1 in plants is a relatively large protein (≈ 940 aa), and the MTase domain is located at its C terminus (Fig. S1 Top). In vitro biochemical assays using the purified *Arabidopsis thaliana* Hen1 shows that the substrate is a small double-stranded RNA (21–24 nt) with a 2-nt overhang at the 3' ends (12). Small single-stranded RNAs, regardless of their sizes, are not the substrate of *A. thaliana* Hen1 (12). Hen1 in animals (≈ 390 aa) is less than half the size of Hen1 in plants. Also, unlike Hen1 in plants, the MTase domain in the animal Hen1 is located at its N terminus (Fig. S1 Middle). In vitro biochemical assays using the purified *Drosophila melanogaster* Hen1 show that the enzyme is able to carry out methylation on small single-stranded RNAs, but not on small double-stranded RNAs (7, 8).

Hen1 homologs are also found in a subset of bacterial species, but their biological function has not been characterized (13). We have recently demonstrated that bacterial Hen1 forms a stable complex with a second protein named Pnkp, and the Pnkp/Hen1 complex constitutes a bacterial RNA repair and modification system (14). Based on a comparison of their relative sizes, bacterial Hen1 is more similar to Hen1 in animals than to Hen1 in plants (Fig. S1 Bottom). However, amino acid sequence comparison indicates that, beyond the MTase domain, bacterial Hen1 has nothing in common with Hen1 in plants and animals. Therefore, there exist three subfamilies of Hen1.

Among the three enzymes involved in small noncoding RNA generation and maturation, two of them, namely Dicer and Argonaute, have been the subjects of extensive structural studies (13, 15–22). However, no structural information of Hen1 is available to date. Here, we present the crystal structures of the MTase domain of bacterial Hen1. The structures, together with

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 3JWG, 3JWH, 3JWI, and 3JWJ).

¹C.M.C. and C.Z. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: huang@uiuc.edu.

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Fig. 1. Methylation activity of Hen1. (A) Nucleotide sequences of four small single-stranded RNAs used for the methylation assays. (B) Incorporation of ^{14}C -methyl group into small single-stranded RNAs. After in vitro reaction, the RNA products were purified and were resolved in 15% DPAGE. The methylated RNA products were detected by exposing the dried gel to a phosphorimaging plate followed by scanning the image plate with a PhosphorImager system.

biochemical characterization of both human and bacterial Hen1, suggest possible mechanisms of RNA substrate recognition and catalysis carried out by Hen1.

Results and Discussion

Enzymatic Activity of Recombinant Hen1. We cloned, overexpressed, and purified human Hen1 (*HsHen1*), two bacterial Hen1 from *Clostridium thermocellum* and *Anabaena variabilis* (*CtHen1* and *AvHen1*), and the C-terminal MTase domain of

CtHen1 and *AvHen1* (*CtHen1*-C and *AvHen1*-C) (Fig. S2). To examine the enzymatic activity of the recombinant proteins, we carried out methylation assays with *HsHen1*, *CtHen1*, and *CtHen1*-C using small single-stranded RNA as substrates (Fig. 1). All three versions of Hen1 were able to methylate small single-stranded RNAs of 21–30 nt (Fig. 1B). Also, the MTase domain of *CtHen1* is more enzymatically active than its full-length counterpart, as well as *HsHen1* (Fig. 1B, lanes 9–12, and compared with lanes 1–8). These results demonstrate that, with small single-stranded RNAs as substrates, the enzymatic activity of the bacterial Hen1 is indistinguishable from its eukaryotic counterpart. Also, the MTase domain of Hen1 is both necessary and sufficient for RNA substrate recognition and catalysis.

Overall Structure. In addition to the direct overexpression and purification of *CtHen1*-C and *AvHen1*-C described in the previous section, we prepared two more MTase domain of bacterial Hen1 by limited protease digestion of the full-length *CtHen1* and *AvHen1*, for structural studies. These two proteins are named *CtHen1*-Cn and *AvHen1*-Cn, respectively, due to a nick introduced in the insertion domain during limited protease digestion. We crystallized all four versions of the MTase domain of bacterial Hen1 and have solved their structures (Table S1). The asymmetric unit of *CtHen1*-C contains one copy of the protein, whereas the symmetric unit of others (*AvHen1*-C, *CtHen1*-Cn, and *AvHen1*-Cn) contains two copies. Also, *AvHen1*-C only crystallized in the presence of AdoMet. Therefore, the hydrolysis product of AdoMet, *S*-adenosylhomocysteine (AdoHcy), is present in the structure (Fig. S3). Because of its higher resolution, the structure of *CtHen1*-C is used for the structural description unless otherwise stated.

Fig. 2. Conservation of the MTase domain of Hen1 from bacteria to human. Amino acid sequences of the representatives of the MTase domain from three subfamilies of Hen1 (three from each subfamily) were aligned. The conserved residues are boxed in color, with completely conserved residues in magenta, identical residues in yellow, and similar residues in cyan. C.ther, *C. thermocellum*; A.vari, *A. variabilis*; H.aura, *Herpetosiphon aurantiacus*; H.sapi, human; M.musc, mouse; D.eri, zebrafish; A.thal, *A. thaliana*; O.sati, rice; P.pate, *Physcomitrella patens*. Residue number over the alignment corresponds to *CtHen1*. The secondary structure of *CtHen1*-C is depicted above the primary sequence, with α -helices highlighted as cylinders, β -strands as arrows, loops as solid lines, and disordered residues as dotted lines.

The core fold of *Ct*Hen1-C belongs to class I AdoMet-dependent MTase (23), consisting of seven-stranded β -sheet (in the order of 3214576) flanked by helices on both sides of the β -sheet (α Z, α A, α B, and α B' on one side, and α C, α D, and α E on the other) (colored blue in Fig. 3) (24). In addition to the core fold, *Ct*Hen1-C possesses a short helix between β 4 and α D (colored magenta in Fig. 3), and an auxiliary domain inserted between β 5 and α E (colored red in Fig. 3). Structural comparison of *Ct*Hen1-C to other DNA and RNA MTases indicated that these two features (α 1 between β 4 and α D, and an insertion domain between β 5 and α E) are unique to Hen1. However, some small-molecule MTases also possess α 1 and an insertion domain between β 5 and α E, although the structures of their insertion domains are different from the one in Hen1 (24). Dali structural homology search (25) found 599 Hen1 homologies with Z score > 3.0 , but all of them have $< 20\%$ amino acid sequence conservation when compared with the MTase domain of *Ct*Hen1-C. No known DNA and RNA MTases are in the top 50 scores (Z score > 17.2), and 43 of the top 50 are the structures of glycine *N*-methyltransferase. However, structural comparison of *Ct*Hen1-C with a representative of glycine *N*-methyltransferase shows that they are very different enzymes (Fig. S4A). Together, Hen1 appears to be evolutionarily divergent from other known RNA and DNA MTases (Fig. S4 B and C). A similar conclusion was reached by Bujnicki and coworkers (26) based on amino acid sequence alignments.

Structure of the Insertion Domain. To probe conformational flexibility within the MTase domain, we aligned all seven structures of the MTase domain of Hen1 (Fig. 4A). The conformations of the core fold and $\alpha 1$ are well defined (Fig. 4A). In contrast, some residues in the insertion domain are unstructured (Fig. 4A and B). All four structures from *Av*Hen1 (*Av*Hen1-C and *Av*Hen1-Cn) have 12 amino acids disordered in the insertion domain (colored yellow, orange, magenta, and gray in Fig. 4B). Although the three structures from *Ct*Hen1 (*Ct*Hen1-C and *Ct*Hen1-Cn) have less disordered amino acids in the insertion domain (colored red, green, and cyan in Fig. 4B), the observed orientation of these residues is a result of crystal packing. The additional $\alpha 3$ observed in the structure of *Ct*Hen1-C (colored red in Fig. 4B) interacts with residues of the AdoMet-binding pocket of the neighboring molecule in the crystal (Fig. S5). Because *Ct*Hen1-Cn was prepared through limited protease digestion from the full-length protein, it was nicked in the insertion domain. As a result, additional amino acids after $\alpha 2$ in both molecules in the asymmetric unit (colored cyan and green in Fig. 4B) are structured, because they interact with the neighboring molecules during crystal packing (Fig. S6). Based on these analyses, we conclude that, in the absence of RNA substrate, the regions of the insertion domain with defined structure are $\alpha 2$ linked to $\beta 5$ (residues 397–405) and a short loop extended from αE (residues 418–424).

Within the MTase domain, the size of the insertion domain varies the most among three subfamilies of Hen1, and the variation is centered in the region that is structurally disordered (Fig. 2). For example, Hen1 from animals is 2–3 aa smaller than the ones from the bacteria, whereas Hen1 from plants is 12–13 residues bigger (Fig. 2). However, the insertion domain is also one of the two most conserved regions (the other one is $\alpha 1$ and its surrounding loops) (Fig. 2). Structural details provide insight into functional roles of some of the conserved amino acids in the insertion domain (Fig. 4C). N398 and E400 from the conserved ³⁹⁶TPNx⁴⁰⁰E motif, W422 and R424 from the conserved ⁴²⁰FEWxR⁴²⁴ motif, have important structural roles for the insertion domain. The side-chain amide of N398 forms two hydrogen bonds with the main-chain carbonyl groups of G455

Fig. 5. Conservation of the MTase domain of Hen1 and a docking model. A single-stranded RNA tetramer (5'-ACGU-3') was docked into a potential RNA-binding groove in CtHen1-C. The structure of CtHen1-C is represented in surface and colored gray. The most conserved amino acids (colored magenta and yellow in Fig. 2) were mapped onto the structure of CtHen1-C. AdoHcy from the structure of AvHen1-C is depicted in spheres and colored cyan. The methyl group of AdoMet was also modeled and highlighted in black. The RNA is represented in sticks and colored by individual atoms (green for carbon, blue for nitrogen, red for oxygen, and orange for phosphate).

and S456 from the loop between $\beta 6$ and $\beta 7$; the side chains of E400 and R424 form two hydrogen bonds with each other; and the side chain of W422 is part of hydrophobic network (Fig. 4C). The resulting structure of the insertion domain presents the remaining conserved residues in these two motifs (T396, P397, F420, and E421) for possible RNA substrate binding (discussed in later section). Because of the uncertainty of their structures, the possible functions of the three remaining conserved amino acids (N402, R414, and H418) in the insertion domain are unclear. However, based on the nature of these amino acids, they may also be involved in interaction with RNA substrate when a RNA substrate is bound.

Docking Model. To provide insight into possible functions of the conserved amino acids located outside the insertion domain, we mapped the conservation of the MTase domain of Hen1 onto the structure of *Ct*Hen1-C (Fig. 5). In addition to a few residues involved in AdoMet binding (Fig. S3), most of the conserved amino acids reside in a deep groove adjacent to the AdoMet-binding pocket, where the RNA substrate presumably binds (Fig. 5). Based on the amino acid conservation and the shape of the potential RNA-binding groove, also guided by the position of the methyl group in AdoMet, we constructed a docking model, in which a single-stranded RNA tetramer (5'-ACGU-3') was manually docked into the structure of *Ct*Hen1-C (Fig. 5).

The docking model places the 3'-terminal nucleotide U4, where the methylation takes place, in a pocket near AdoMet. Two conserved amino acids from the insertion domain, F420 and E421, form the upper wall of the pocket (Fig. 6A). Three conserved amino acids, one (P397) from the insertion domain and two (E369 and H370) from *a1*, provide the side wall of the pocket (Fig. 6A). And the side chains of R273 and E366 are the floor of the pocket (Fig. 6A). The length of the RNA-binding groove appears to be able to accommodate three to four nucleotides of RNA (Fig. 5). Based on the model, the side chain

Fig. 6. Methylation activities of Hen1 mutants. (A) Predicted detailed interactions between RNA substrate and CtHen1-C according to the docking model. Protein is depicted and colored as in Fig. 4C except the amino acids outside the insertion domain, which are colored orange to provide some distinction from the ones from the insertion domain. RNA and AdoMet are depicted in sticks and are colored as in Fig. 5 except the phosphate atoms, which are colored magenta to distinguish them from the side chains of the protein. (B) DPAGE analysis of RNA products purified from the methylation reactions to determine the enzymatic activity of CtHen1-C mutants. (C) Quantitative analysis of the methylation activity of CtHen1-C mutants shown in B. The relative activity of each mutant was determined by comparing the amount of radioactivity incorporated into RNA substrate by the mutant with that incorporated by the WT CtHen1-C, with the relative activity of the wild-type enzyme normalized to 100%.

of the strictly conserved L269 from αZ divides the RNA tetramer equally, with two nucleotides inside and two outside (Fig. 5). The two nucleotides away from the active site can also be in duplex form without steric clash with the enzyme (Fig. S7), predicting that a double-stranded RNA with at least 2-nt 3'-overhang could also be the substrate of the enzyme.

Mutational Studies. The docking model allows us to predict the possible functions of some conserved amino acids. In addition to the insertion domain, the contribution of RNA substrate binding and catalysis are predicted from three regions: $\alpha 1$ and its surrounding loops, the top of the N-terminal helix αZ , and the tip of the last β strand (Fig. 6A).

The consensus sequence of $\alpha 1$ and its surrounding loops is $^{366}\text{EhhEHhD}/\text{E}^{372}$ (where h is L, V, I, or M; Fig. 2). According to the docking model, the side chains of H370, E369, and E366 are in hydrogen bond distance with the 2'-OH group of U4, the 3'-OH group of U4, and the 2'-OH group of G3, respectively (Fig. 6A). These conserved amino acids are critical to Hen1, because alanine mutant of each of them abolished the enzymatic

activity (Fig. 6B and C, lanes 5–7). Among these three, H370 is likely to be the general base because of its possible hydrogen bonding to the 2'-OH group of U4 where the methylation takes place.

The N terminus of the MTase domain of Hen1 has a conserved motif with the consensus sequence of $^{269}\text{LxxQR}^{273}$ (Fig. 2). The side chains of R273 and Q272 are predicted to interact with the phosphate groups of U4 and C2, respectively (Fig. 6A). If these predictions are correct, R273 should be more important than Q272, because R273 interacts with the phosphate group of U4 where the methylation takes place. Indeed, R273A mutant has no detectable enzymatic activity (Fig. 6B and C, lane 4). However, Q272A mutation maintains 69% activity of the WT enzyme (Fig. 6B and C, lane 3). In fact, Q272 is not strictly conserved when a larger pool of Hen1 sequences is used for the alignment, consistent with its nonessential role as indicated by the mutational study. Perhaps the most interesting conservation at the N terminus is L269. The side chain of L269 lacks a functional group and is hydrophobic in nature. The docking model shows the side chain of L269 stacks on the base of G3 (Fig. 6A). Therefore, it appears to have a role to secure the position of the two 3'-terminal nucleotides near the active site of the enzyme for methylation. L269A mutation reduces the enzymatic activity of Hen1 to 4% of the WT enzyme (Fig. 6B and C, lane 2).

The consensus sequence of the C terminus is $^{458}\text{T/SQhG}/\text{AhF}^{463}$ (where h is M, L, I, or V; Fig. 2). Q459 and F463 have structural roles to secure the position of $\beta 7$, allowing the side chain of T458 to be presented for possible hydrogen bond with the phosphate group of G3 (Fig. 6A). Only 4% enzymatic activity remains with the T458A mutation (Fig. 6B and C, lane 12).

We have previously discussed structural roles of four conserved amino acids from the two conserved motifs ($^{396}\text{TPNx}^{\text{E}400}$ and $^{420}\text{FEWxR}^{424}$) in insertion domain (Fig. 4C). The remaining four (T396, P397, F420, and E421) may be involved in RNA substrate binding and catalysis (colored yellow in Fig. 6A). The side chain of T396 forms a hydrogen bond with the conserved E369 from $\alpha 1$, which may interact with the 3'-OH of the targeted ribose. T396A mutation abolished the enzymatic activity of CtHen1-C (Fig. 6B and C, lane 8). P397 and the side chain of F420 may be part of the binding pocket for the 3'-terminal nucleotide of RNA substrate. P397A mutant has no detectable enzymatic activity, and only 1.5% enzymatic activity remains with F420A mutation (Fig. 6B and C, lanes 9 and 10). E421 points toward the potential RNA-binding cleft and may also interact with some part of RNA substrate, and E421A mutant has no detectable enzymatic activity (Fig. 6B and C, lane 11).

The studies reported here clearly demonstrate that Hen1 from three different subfamilies carry out the same chemical reaction: to methylate the 2'-OH group at the 3'-terminal nucleotide of RNA. However, the biological functions of Hen1 appear to be distinct based on our recent studies. The eukaryotic Hen1 is involved in RNAi. However, we have recently shown that the bacterial Hen1 is involved in RNA repair (14). The same chemical reaction, but distinct biological functions of Hen1 raises an intriguing question in terms of the possible evolutionary origin of Hen1. With the exception of the MTase domain, Hen1 in plants, animals, and bacteria have nothing in common in terms of domain arrangements and protein size (Fig. S1). In fact, the MTase domains of plant Hen1 and animal Hen1 are located at the opposite ends of the proteins (C terminus vs. N terminus). Given that the other two major players of RNAi, Dicer and Argonaute, are well conserved between plants and animals, the drastic divergence of Hen1 in these two classes of eukaryotes might imply more recent functional acquisition of Hen1 in RNAi. If this is the case, it is possible that the methylation step involved in eukaryotic small noncoding RNA maturation might have been borrowed from other organisms possessing more

ancient Hen1 via horizontal gene transfer. It remains to be seen whether a bacterial species possessing Pnkp/Hen1 heterotetramer is the source of such horizontal gene transfer for eukaryotic Hen1, or both the bacterial and eukaryotic Hen1 are from a common and more ancient organism (possibly a virus) whose identity has yet to be revealed.

Materials and Methods

Recombinant Proteins. Cloning, expression, and purification of Hen1 are described in *SI Materials and Methods*.

Crystallography. Crystallization, data collection, and structural determination of Hen1 are described in *SI Materials and Methods* and Table S1.

Methylation Assays. The methylation assays were carried out in a reaction mixture of 20- μ L scale containing 25 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, 0.05 mM EDTA, 2.5% glycerol, 5 mM DTT, 2 mM MnCl₂, 20 μ M ¹⁴C-AdoMet, and 10 μ M single-stranded RNA/5 μ M protein. The reaction mixtures were incubated at 37 °C for 40 min. Phenol extraction was carried out after the reaction. The aqueous layer was recovered, and RNA was purified by ethanol precipitation. The purified RNA was dissolved in 10 μ L of TE buffer, and 10 μ L of denaturing PAGE (DPAGE) loading buffer was added. The sample was heated at 95 °C for 2 min and then analyzed by a 15% DPAGE.

The gel was dried and exposed to a phosphorimaging plate, and the radioactivity of RNA was detected with a PhosphorImager system.

Construction of a Docking Model of RNA–Hen1 Complex. The structure of RNA tetramer with the nucleotide sequence of 5'-ACGU-3' was generated by using the program Coot (27). The RNA tetramer was then manually docked into the potential RNA-binding cleft in CtHen1-C by using PyMOL (28). The position of each nucleotide was adjusted to optimize the fitting of RNA with the shape of the potential RNA-binding groove in CtHen1-C.

Mutational Studies. The CtHen1-C mutants were created through QuikChange using the overexpression vector of the WT enzyme as the template and the primers purchased from Integrated DNA Technologies. The mutants were overexpressed in *Escherichia coli* and purified individually with Ni²⁺ affinity and Heparin columns analogous to the purification of the WT enzyme. The purified mutants were concentrated to $\approx$ 6 mg/mL for methylation assays or storage. The methylation assays of mutants were carried out similar to the ones for small single-stranded RNAs with different length shown in Fig. 1, except the scale of the reaction was increased 3-fold to enhance the sensitivity of the assays.

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