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Unique 2′-O-Methylation by Hen1 in Eukaryotic RNA Interference and Bacterial RNA Repair

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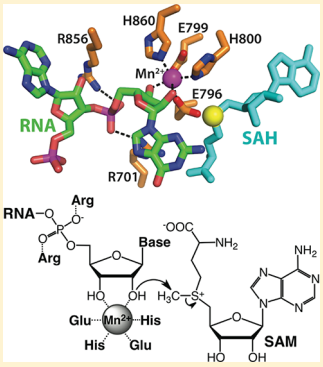
ABSTRACT: In an RNA transcript, the 2′-OH group at the 3′-terminal nucleotide is unique as it is the only 2′-OH group that is adjacent to a 3′-OH group instead of a phosphate backbone. The 2′-OH group at the 3′-terminal nucleotide of certain RNAs is methylated in vivo, which is acheived by a methyltransferase named Hen1 that is mechanistically distinct from other known RNA 2′-O-methyltransferases. In eukaryotic organisms, 3′-terminal 2′-O-methylation of small RNAs stabilizes these small RNAs for RNA interference (RNAi). In bacteria, the same methylation during RNA repair results in repaired RNA resisting future damage at the site of repair. Although the chemistry performed by the eukaryotic and bacterial Hen1 is the same, the mechanisms of how RNA is stabilized as a result of the 3′-terminal 2′-O-methylation are different between the eukaryotic RNAi and the bacterial RNA repair. In this review, I will discuss the distribution of Hen1 in living organisms, the classification of Hen1 into four subfamilies, the structure and mechanism of Hen1 that allows it to conduct RNA 3′-terminal 2′-O-methylation, and the possible evolutionary origin of Hen1 present in bacterial and eukaryotic organisms.



The fundamental difference between RNA and DNA is the 2′-OH groups. Because of the presence of the 2′-OH groups in RNA, a double-stranded RNA is in an A form whereas a double-stranded DNA is in a B form. More importantly, the 2′-OH groups in an RNA facilitate the folding of the RNA into a tertiary structure, resulting in most RNAs having biological functions more like those of proteins than those of DNA. From a chemical point of view, however, the 2′-OH groups make RNA more susceptible to degradation. A 2′-OH group in an RNA can conduct an intramolecular nucleophilic attack on its adjacent phosphate backbone, resulting in RNA cleavage. An overwhelming majority of RNA cleavage occurring in vivo adopts this transesterification mechanism, regardless of whether the cleavage is spontaneous or enzymatically catalyzed.

Methylation of a 2′-OH group in an RNA (RNA 2′-O-methylation) enhances the stability of the RNA because it eliminates the potential of the 2′-OH group as a nucleophile for RNA cleavage. Indeed, many methyltransferases have been found in nature to conduct site-specific RNA 2′-O-methylation.[1−8] In addition to protein enzymes, there is even a family of ribonucleoprotein complexes named C/D box snoRNA that performs RNA 2′-O-methylation using the RNA component of the complex as the guide for recognition of the methylation site.[9,10] Among the many RNA modifications occurring in vivo, RNA 2′-O-methylation is one of the two most abundant modifications (with the formation of pseudouridine being the other).

The underlying principle of RNA stabilization via 2′-O-methylation described above applies to all 2′-OH groups in an RNA except one, the 2′-OH group at the 3′-terminal nucleotide. Unlike other 2′-OH groups that are adjacent to a phosphate backbone, the neighbor of the 2′-OH group at the 3′-terminal nucleotide is the 3′-OH group. Therefore, it is not straightforward to envision how the methylation of the 2′-OH group at the 3′-terminal nucleotide of an RNA could stabilize the RNA as it does not have a nearby RNA cleavage target. However, a unique methyltransferase named Hen1, first found in a eukaryotic organism[11] and later found in bacteria,[12] specifically methylates the 2′-OH group at the 3′-terminal nucleotide of RNAs, and the main biological function of Hen1 is indeed to enhance the stability of an RNA via 3′-terminal 2′-O-methylation. In this review, I will discuss several aspects of RNA 3′-terminal 2′-O-methylation by Hen1, including the distribution of Hen1 in living organisms, the classification of Hen1 into four subfamilies, the biological functions of Hen1, the underlying mechanism of 2′-O-methylation conducted by Hen1, and the possible evolutionary origin of Hen1.

■ DISTRIBUTION OF HEN1 IN LIVING ORGANISMS

Hen1 was first discovered in *Arabidopsis thaliana* as a methyltransferase that specifically methylates the 2′-OH group at the 3′-terminal nucleotide of small RNAs.[11] Subsequently, Hen1 homologues were found to conduct the same chemical reaction in members of the animal kingdom.[13−15] More recently, bacterial Hen1 was shown to be involved in RNA repair.[12] To provide an overview of the distribution of Hen1 in living organisms, bioinformatic analysis of Hen1 was performed. Specifically, a search for amino acid sequences

Received: April 17, 2012
Revised: May 3, 2012
Published: May 7, 2012

dx.doi.org/10.1021/bi300497x | *Biochemistry* 2012, 51, 4087−4095

similar to that of human Hen1 (*Hsa*Hen1) using the BLAST program (Blastp) was conducted, yielding the sequences of all available forms of Hen1.[16] After some incomplete sequences were manually deleted, the retrieved Hen1 sequences were subject to pairwise Blastp, and the results were displayed as a sequence similarity network using Cytoscape (Figure 1).[17]

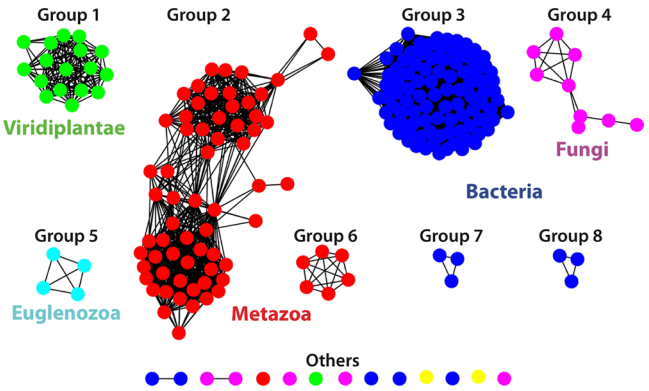


Figure 1. Visualization of the distribution of Hen1 in different branches of living organisms. The results of a pairwise Blastp search of Hen1 sequences were displayed as a sequence similarity network in Organic layout using Cytoscape. Each node (colored cycle) represents an individual Hen1. An edge (black line) connects two nodes if the *e* value measuring the sequence similarities of Hen1 that these two nodes represent is smaller than a cutoff value, which was set as 1×10^{-50} for this particular network. Forms of Hen1 from bacteria are colored blue, and the remaining forms of Hen1 are all from eukaryotic organisms. Eukaryotic forms of Hen1 are colored on the basis of the kingdoms of the organisms, with Viridiplantae colored green, Metazoa red, Fungi magenta, Euglenozoa cyan, and Alveolata yellow. The set of Hen1 sequences for the network was based on a Blastp search of human Hen1. The sequence similarity networks constructed from the sets of Hen1 sequences based on a Blastp search of the MTase domain of plant Hen1 and a bacterial Hen1 produced similar results.

Hen1 is found in organisms from eukaryota and bacteria, but not from archaea. The sequence similarity network divides forms of Hen1 into eight clusters (Figure 1, groups 1−8) as well as some unclustered Hen1 (Figure 1, others). However, if the classification of Hen1 is based on the arrangements of the domains in Hen1, the entire population of Hen1 can be grouped into four subfamilies (Figure 2A). Because of the strong correlation between a subfamily and the biological function of Hen1 shared by the members within the subfamily, my discussion of Hen1 will be based on classification of Hen1 as four subfamilies with additional incorporation of data from the sequence similarity network shown in Figure 1.

The first subfamily of Hen1 includes 18 organisms from the Viridiplantae kingdom (Figure 1, colored green). With one exception, all of them are classified as group 1 and belong to the Streptophyta phylum. Two features define a member of forms of Hen1 in group 1. First, it is a relatively large protein, with the size of the protein ranging from 810 to 970 amino acids (aa) (Figure 2A, first). Second, the methyltransferase (MTase) domain, which is approximately 200 aa in size, is located at the C-terminus (Figure 2A, first). The exception is Hen1 from *Volvox carteri* (*Vca*Hen1), which belongs to the Chlorophyta phylum and is classified as others instead of group 1 (Figure 1, others, colored green). *Vca*Hen1 is a 1595 aa protein. Although the MTase domain is also located at the C-terminus like that of Hen1 in group 1, the remainder of *Vca*Hen1 has no significant sequence similarities with *Ath*Hen1. Therefore, it is possible that Hen1 of the Chlorophyta phylum might be different from those of the Streptophyta phylum.

The remaining eukaryotic forms of Hen1 all belong to the second subfamily (Figure 2A, second). They include Hen1 from 65 species of Metazoa (Figure 1, colored red), 14 species of Fungi (Figure 1, colored magenta), four species of Euglenozoa (Figure 1, colored cyan), and two species of Alveolata (Figure1, colored yellow). The unique feature of this subfamily of Hen1 is that the MTase domain is located at the N-terminus (Figure 2A, second). However, the C-terminal domain of this subfamily of Hen1 varies in size and is less conserved, or not conserved at all. The overwhelming majority of forms of Hen1 from organisms of Metazoa belong to group 2 (Figure 1). Two exceptions are Hen1 from six organisms of the worm species, including the model organism *Caenorhabditis elegans* (Figure 1, group 6), and Hen1 from *Oikopleura dioica* that belongs to the Chordata phylum (Figure 1, others and colored red). Nine of 14 forms of Hen1 from Fungi belong to group 4, and the remaining five are unclustered (Figure 4, others and colored magenta). Forms of Hen1 from four species of Euglenozoa, including the model organism *Trypanosoma brucei*, are in group 5. Finally, Hen1 from two species of Alveolata, including the model species of *Tetrahymena thermophila*, are also unclustered (Figure 1, others and colored yellow).

The third subfamily includes bacterial forms of Hen1 that are part of a Pnkp/Hen1 RNA repair system (Figure 2A, third). Like that of Hen1 of the first subfamily, the MTase domain of Hen1 of the third subfamily is also located at the C-terminus. However, there are no sequence similarities in the N-terminal part of Hen1 between the first subfamily and the third subfamily. The overwhelming majority (a total of 91 species) of the third subfamily belongs to group 3 (Figure 1). However, we have recently discovered forms of Hen1 from three additional bacterial species also involved in RNA repair, but they are significantly different in amino acid sequence from the ones that belong to group 3 (unpublished results). The newly discovered bacterial forms of Hen1 were classified as a separate cluster by the sequence similarity network (Figure 1, group 7).

The fourth subfamily contains forms of Hen1 from eight bacterial species that have only the MTase domain (Figure 2A, fourth). They include forms of Hen1 from three organisms that belong to group 8 and five organisms that are unclustered (Figure 1, others and colored blue). Unlike forms of Hen1 of the first three subfamilies, no study has been reported on Hen1 of the fourth subfamily.

BIOLOGICAL FUNCTIONS OF HEN1

As mentioned previously, Hen1 was first discovered in *Arabidopsis* to conduct 2′-O-methylation at the 3′-terminal nucleotide of siRNAs and miRNAs.[11] Subsequent in vitro biochemical characterization of *Ath*Hen1 showed the preferred RNA substrate of *Ath*Hen1 is a small RNA duplex of 21−24 nucleotides (nt) with a two-nucleotide 3′-overhang.[18] The crystal structure of *Ath*Hen1 in complex with a 22 nt small RNA duplex provides the molecular explanation of the observed substrate specificity.[19] Whereas the MTase domain at the C-terminus of *Ath*Hen1 is involved in catalysis, the four remaining domains in *Ath*Hen1, dsRBD1, LCD, dsRBD2, and PLD (Figure 2A, first), are responsible for specific recognition and binding of a double-stranded RNA of 21−24 nt.[19]

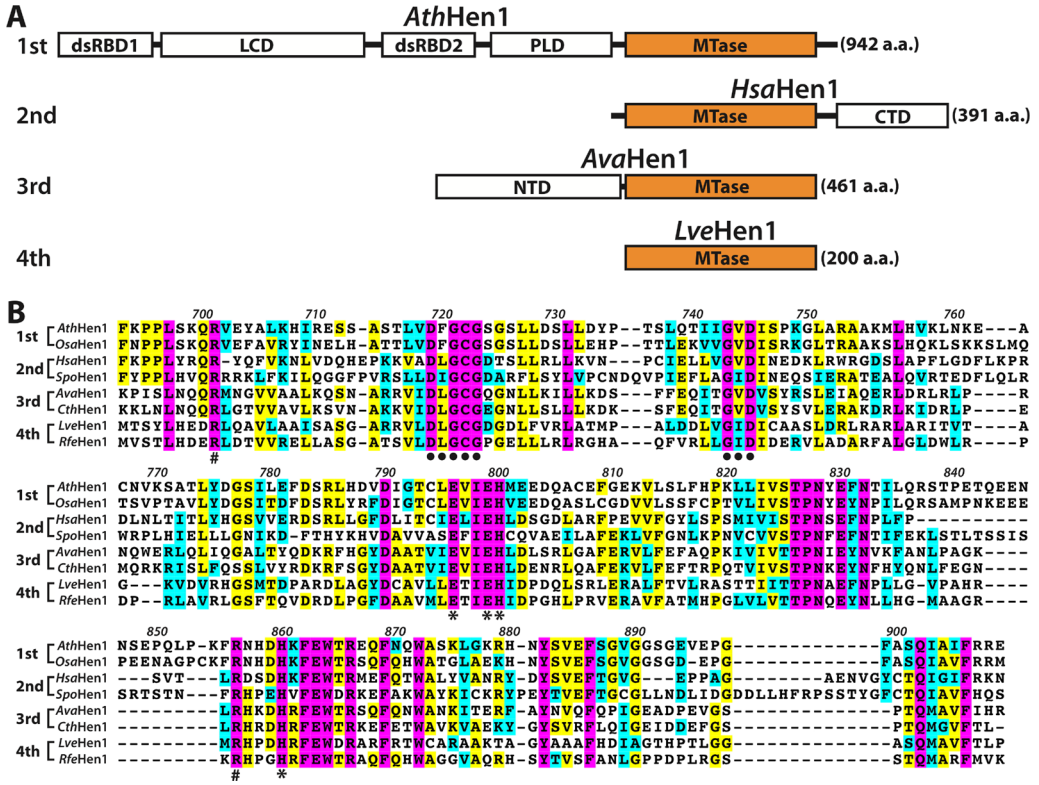


Figure 2. Classification of Hen1 into four subfamilies and the conservation of the shared MTase domain in Hen1. (A) Schematic representation of the domain architectures of four forms of Hen1, each of which represents a subfamily. The shared MTase domain of Hen1 is vertically aligned and highlighted in orange. Abbreviations: *Ath*, *A. thaliana*; *Hsa*, *Homo sapiens*; *Ava*, *Anabaena variabilis*; *Lve*, *Loktanella vestfoldensis*; dsRBD, double-stranded RNA binding domain; LCD, La motif-containing domain; PLD, PPIase-like domain; CTD, C-terminal domain; NTD, N-terminal domain. (B) Sequence alignments of the MTase domain of eight forms of Hen1, with two representatives from each subfamily. The conserved residues are boxed in color, with completely conserved residues colored magenta, identical residues yellow, and similar residues cyan. The residue number over the alignments corresponds to *Ath*Hen1. Residues involved in SAM binding are marked with black dots. Four conserved residues responsible for coordinating a metal ion are marked with asterisks, and two conserved residues interacting with the phosphate backbone of the 3′-terminal nucleotide of RNA are marked with number signs. Abbreviations: *Osa*, *Oryza sativa*; *Spo*, *Schizosaccharomyces pombe*; *Cth*, *Clostridium thermocellum*; *Rfe*, *Rhodoferax ferrireducens*.

The 3′-terminal 2′-O-methylation of siRNAs and miRNAs by *Ath*Hen1 enhances their stability in vivo. This was first demonstrated in *Arabidopsis* mutants with defective *Ath*Hen1 that have a reduced abundance of miRNAs and increased heterogeneity in the size of miRNAs.[11] The cause of the heterogeneity was found to be the addition of uridines (uridylation) at the 3′-ends of small RNAs in the absence of 2′-O-methylation (Figure 3, left). The 3′-terminal 2′-O-methylation by *Ath*Hen1 blocked the uridylation reaction (Figure 3, right).[20] The enzyme responsible for uridylation in *Arabidopsis* was recently discovered, and addition of poly(U) promotes degradation of the marked RNAs (Figure 3).[21,22]

In most animal species, siRNAs and miRNAs are not 2′-O-methylated at their 3′-ends. Instead, a third class of small RNA, piRNA, is 2′-O-methylated at the 3′-terminal nucleotide, and the methylation is conducted by Hen1 of the second subfamily (Figure 2A, second).[13−15] Unlike siRNAs and miRNAs, which are double-stranded and are generated by Dicer, piRNAs are single-stranded and are produced by Piwi proteins via a ping-pong mechanism.[23,24] Hen1 of the second subfamily was shown to physically interact with Piwi proteins, presumably via the C-terminal domain (Figure 2A, CTD).[13,25] Therefore, the enzymatic reactions of generating piRNAs by Piwi proteins and 3′-terminal 2′-O-methylation by Hen1 are likely to be coupled events.

As in the case of plant small RNAs, 2′-O-methylation of piRNAs blocks uridylation at their 3′-ends, preventing them from degradation (Figure 3).[26,27] Therefore, although forms of Hen1 from the first and the second subfamilies are significantly different in domain architectures and RNA substrate specificity, the mechanism of how small RNAs are stabilized due to 3′-terminal 2′-O-methylation by Hen1 appears to be the same.

Soon after the discovery of *Ath*Hen1, Tkaczuk et al. conducted bioinformatic analyses of Hen1 and showed the presence of Hen1 in bacteria.[28] Further bioinformatic analyses of bacterial Hen1 conducted in my laboratory revealed that the overwhelming majority of bacterial forms of Hen1 are 460−500 aa in size and possess a domain structure of the third subfamily as shown in Figure 2A. Furthermore, we found that the bacterial species having the gene encoding Hen1 of the third subfamily always possess a second gene encoding Pnkp (polynucleotide kinase phosphatase), and both of them are in the same operon. Martins and Shuman showed that the bacterial Pnkp from *Clostridium thermocellum* possesses kinase, phosphatase, and adenylyltransferase activities.[29] We demonstrated that the bacterial Pnkp and Hen1 from *Anabaena variabilis* form a stable complex in vitro (Figure 4A), and the complex was able to repair tRNAs cleaved by ribotoxins.[12] On the basis of our study as well as studies by Shuman and co-

dx.doi.org/10.1021/bi300497x | *Biochemistry* 2012, 51, 4087−4095

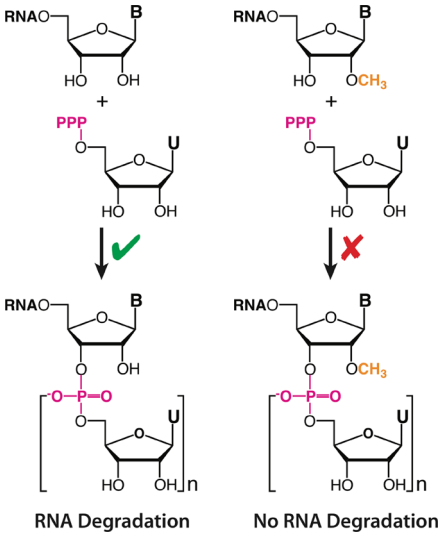


Figure 3. Biological consequence of 3′-terminal 2′-O-methylation of small RNAs by the eukaryotic Hen1. The fate of a small RNA in vivo depends on the status of 2′-O-methylation at its 3′-terminal nucleotide. If the 2′-OH group is unmethylated, uridines can be added by a terminal nucleotidyl transferase in a template-independent manner on the 3′-end of the small RNA, leading the marked RNA to degrade (left). Hen1 methylates the 2′-OH group at the 3′-terminal nucleotide of a small RNA, resulting in inhibition of uridylation and thus stabilizing the RNA (right).

workers, a mechanism of RNA repair conducted by bacterial Pnkp/Hen1 can be proposed as shown in Figure 4B.

A classical RNA repair, exemplified by the first discovered RNA repair system from bacteriophage T4,[30] requires three essential enzymatic steps: (i) 5′-phosphorylation by a kinase, (ii) 3′-dephosphoylation by a phosphatase, and (iii) ligation of the two processed ends by a ligase. The bacterial Pnkp/Hen1 system is distinguished from other RNA repair systems by addition of a fourth enzymatic activity, 2′-O-methylation at the 3′-terminal nucleotide of the cleaved RNA during RNA repair (Figure 4B, colored orange). Therefore, unlike other RNA repair systems that restore the cleaved RNA to its original form, an RNA repaired by the bacterial Pnkp/Hen1 system acquires an extra methyl group attached to the 2′-OH group that is responsible for the original RNA cleavage. Thus, the biological function of bacterial Hen1 is to prevent the repaired RNA from cleaving again at the site of repair.[12]

It is interesting to point out that, from a chemical point of view, the site of the repaired RNA with a combination of 3′-terminal 2′-O-methylation and RNA ligation is indistinguishable from a single enzymatic step of internal 2′-O-methylation of an intact RNA (Figure 4B, bottom repaired product). Therefore, the mechanism of RNA stabilization due to 2′-O-methylation by bacterial Hen1 is traditional: Methylation eliminates the potential of the 2′-OH group to become a nucleophile to cleave its adjacent phosphate backbone. From an RNA repair point of view, however, the only way to achieve the absolute substrate specificity of 2′-O-methylation is the sequential enzymatic reactions of 3′-terminal 2′-O-methylation followed by RNA

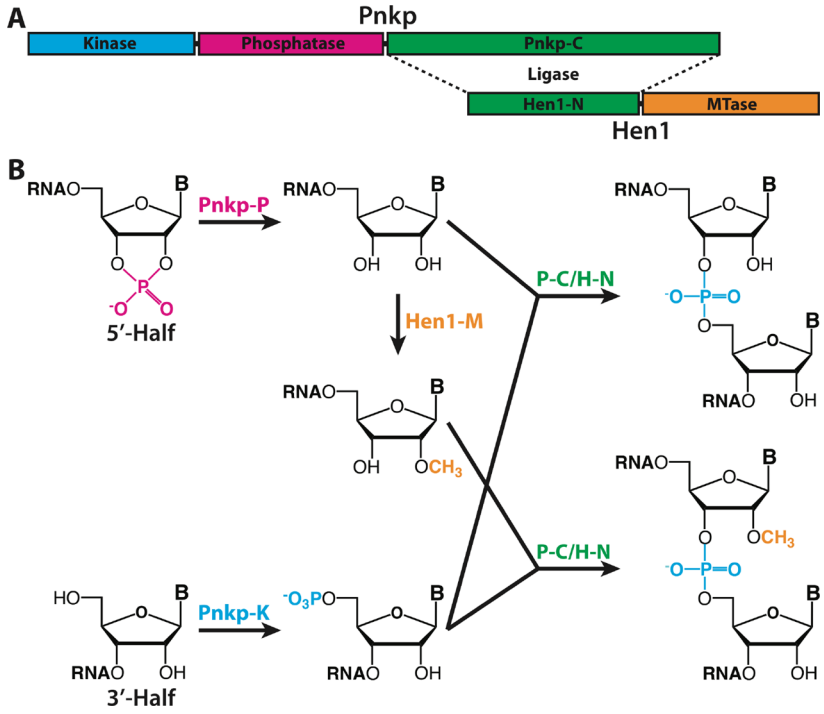


Figure 4. Biological consequence of 3′-terminal 2′-O-methylation of damaged RNAs by the bacterial Hen1. (A) Schematic representation of the bacterial Pnkp/Hen1 complex involved in RNA repair and modification. Domains are colored on the basis of enzymatic activities, with kinase colored cyan, phosphatase magenta, ligase green, and methyltransferase orange. Dashed lines between the C-terminal half of Pnkp (Pnkp-C) and the N-terminal half of Hen1 (Hen1-N) denote physical association between them as well as the requirement of both for RNA ligation. (B) Schematic view of the enzymatic reactions on the two ends of a cleaved RNA (5′-Half and 3′-Half) by bacterial Pnkp/Hen1 that lead to RNA repair. In vitro study showed that both unmethylated (top) and methylated (bottom) repaired RNA products were produced by bacterial Pnkp/Hen1, depending on whether the 2′-OH group at the 3′-terminal nucleotide of the cleaved RNA is methylated during RNA repair. Abbreviations: Pnkp-K, Pnkp kinase; Pnkp-P, Pnkp phosphatase; Hen1-M, Hen1 MTase; P−C, Pnkp C-terminal half; H−N, Hen1 N-teminal half.

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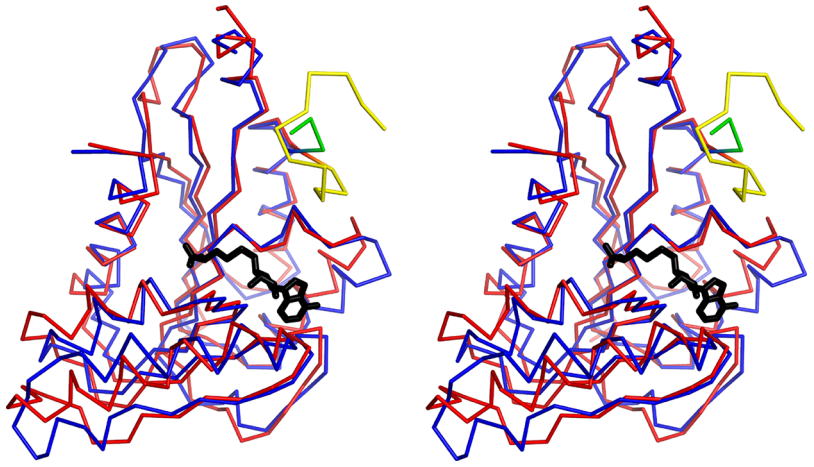

Figure 5. Structural conservation of the MTase domain of Hen1. Stereoview of the superposition of the Cα atoms of the structures of the MTase domain of *Ath*Hen1 (red) with that of *Ava*Hen1 (blue). The root-mean-square deviation (rmsd) of the two structures is 1.4 Å. The bound SAH is depicted as black sticks to provide the location of the SAM-binding pocket in Hen1. The region of a significant structural change in *Ath*Hen1, presumably resulting from RNA substrate binding, is colored yellow, and its corresponding region in *Ava*Hen1 is colored green or disordered.

ligation. As soon as the cleaved RNA is ligated, it is too late to attempt to locate and methylate the 2′-OH group that is responsible for the original RNA cut because it becomes indistinguishable from other 2′-OH groups in the RNA after ligation.

The biological functions of Hen1 of the fourth subfamily are unknown as no study has been reported. Chemically, forms of Hen1 from the fourth subfamily are likely to conduct the same 3′-terminal 2′-O-methylation of RNA because the amino acids essential for catalysis are conserved in forms of Hen1 from the fourth subfamily (Figure 2B).

MECHANISM OF RNA 3′-TERMINAL 2′-O-METHYLATION BY HEN1

Structural and biochemical studies of Hen1 provide insight into the mechanism of 3′-terminal 2′-O-methylation conducted by the MTase domain of Hen1.[19,31−34] The crystal structure of *Ath*Hen1 in complex with a small RNA duplex and *S*-adenosylhomocysteine (SAH),[19] together with several structures of the MTase domain of bacterial Hen1,[31] revealed that the core fold of the MTase domain of Hen1 belongs to class I *S*-adenosylmethionine (SAM)-dependent methyltranferases,[35,36] consisting of a seven-stranded β-sheet flanked by helices on both sides of the β-sheet. The structural studies of forms of Hen1 from different subfamilies confirmed the conservation of the MTase domain of Hen1 among the subfamilies (Figure 5).

The structural study of the *Ath*Hen1−RNA−SAH ternary complex is particularly significant as it provides insight into the mechanism of catalysis by Hen1.[19] Specifically, the structure revealed the presence of a metal ion in the active site. The metal ion is coordinated by the 2′-OH and 3′-OH groups of the terminal nucleotide of the RNA substrate as well as the side chains of four amino acids from Hen1 (Figure 6). The requirement of a metal ion for catalysis by Hen1 was further supported by subsequent biochemical assays as depletion of the metal ion by EDTA in the reaction mixture abolished the enzymatic activity of Hen1.[19,33] Therefore, unlike other known RNA 2′-O-methyltransferases, Hen1 conducts 2′-O-methylation via a metal ion-dependent mechanism. The four residues (two glutamates and two histidines) coordinating the metal ion are strictly conserved among four subfamilies of Hen1 (Figure 2B).

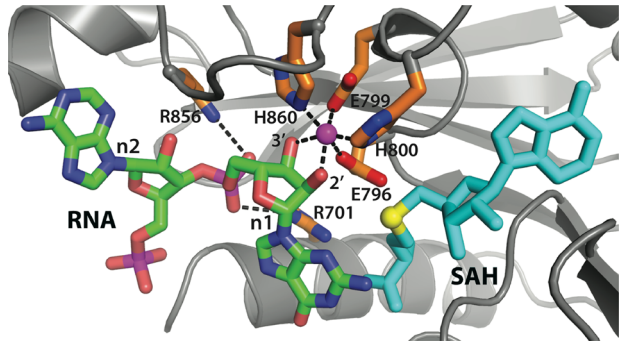

Figure 6. Metal ion-dependent catalysis of RNA 3′-terminal 2′-O-methylation conducted by Hen1. Details of the active site based on the structure of *Ath*Hen1 in complex with RNA and SAH. The Cα atoms of Hen1 are shown as gray ribbons. RNA, SAH, and the side chains of six conserved residues of Hen1 are shown as sticks. RNA is colored green except heteroatoms, which are colored individually (blue for nitrogen, red for oxygen, and magenta for phosphate). SAH is colored cyan, except the sulfur atom, which is colored yellow and depicted as a sphere to highlight the location of the missing methyl donor. The side chains of Hen1 are colored orange, except the heteroatoms, which are also colored individually (blue for nitrogen and red for oxygen). A metal ion is depicted as a magenta sphere. Metal ion coordinations and salt bridges are depicted with black dashed lines. Only the two nucleotides of RNA that are closet to the active site are shown for the sake of clarity.

In addition to these four residues, the side chains of two arginines interact with the phosphate backbone of the 3′-terminal nucleotide (Figure 6), presumably orienting the targeted nucleotide for catalysis. These two arginines are also strictly conserved in Hen1 (Figure 2B). Thus, the six strictly conserved residues (two glutamates, two histidines, and two arginines) are likely to be the signature feature of the MTase domain of Hen1.

Structures of Hen1 with and without RNA bound also provide insight into the dynamic nature of the active site assembly. In the absence of the RNA substrate, no metal ion was found in the active site of the MTase domain of bacterial Hen1 despite the presence of SAH in some of the structures.[31] Structural comparison of the MTase domain of *Ath*Hen1 (with RNA bound) and *Ava*Hen1 (without RNA bound) showed

dx.doi.org/10.1021/bi300497x | *Biochemistry* 2012, 51, 4087−4095

significant conformational change in the loop possessing the conserved R856 and E860 in *Ath*Hen1 and their equivalence in *Ava*Hen1 (Figure 5, colored yellow and green). While the loop in *Ath*Hen1 is structured (with R856 and E860 involved in RNA and metal ion binding, respectively), most residues in the equivalent loop in *Ava*Hen1 are disordered (Figure 5, colored green). This structural difference, together with the absence of a metal ion in all seven structures of the MTase domain of bacterial Hen1, indicates that the coordination of a metal ion in the active site is a dynamic process and requires both the side chains of four strictly conserved amino acids from Hen1 and the two OH groups (2′ and 3′) from the 3′-terminal nucleotide of the RNA substrate.

The requirement of a metal ion for catalysis by Hen1 neatly addresses the substrate specificity issue of 2′-O-methylation at the 3′-terminal nucleotide of RNA. If the assembly of a metal ion in the active site of Hen1 requires all six ligands, only the 3′-terminal nucleotide of an RNA is capable of conducting the assembly because it is the only nucleotide in the RNA that possesses the 2′- and 3′-OH groups. All other nucleotides in the RNA are not capable of this because they either lack the 3′-OH group and/or the neighborhood of the 2′-OH group is too bulky (because of the presence of the adjacent phosphate backbone).

Although a metal ion is undoubtedly required for catalysis by Hen1,[19,33] uncertainty remains in terms of the identity of the metal ion. The structural study of the *Ath*Hen1−RNA−SAH ternary complex assigned Mg^{2+} as the coordinated ion in the active site, and the supplemental biochemical data supported the Mg^{2+}-dependent catalysis by *Ath*Hen1.[19] On the other hand, Jain et al. showed the requirement of Mn^{2+} for catalysis by the bacterial Hen1 from *C. thermocellum* and the enzyme was inactive with Mg^{2+}.[33] Our studies using forms of Hen1 from several bacterial species confirmed the results of Jain et al. (unpublished results). Furthermore, our study using the purified recombinant human Hen1 also showed the requirement of Mn^{2+} for catalysis (unpublished results). Therefore, further studies of *Ath*Hen1 or other forms of Hen1 of the first subfamily are required to provide additional data to address whether the metal ion requirement by Hen1 of the first subfamily is indeed different from those of the second and third subfamilies, or if Hen1 of the first subfamily also requires Mn^{2+} for catalysis.

■ POSSIBLE EVOLUTIONARY ORIGIN OF HEN1

With the exception of forms of Hen1 of the fourth subfamily, forms of Hen1 of three other subfamilies possess extra domains in addition to the MTase domain (Figure 2A). As discussed previously and more below, the presence of additional domains in Hen1 of each subfamily is correlated to a certain biological function of Hen1 from the subfamily, and they are different from one another between subfamilies. Therefore, the gene encoding Hen1 of a subfamily could be evolved from a fusion of the gene encoding the MTase domain and genes encoding the additional domains that are required for the biological function of Hen1 of that particular subfamily.

In plants, siRNAs and miRNAs require 3′-terminal 2′-O-methylation. Dicer is responsible for generating siRNAs and miRNAs in vivo in the form of an RNA duplex of 21−24 nt with a 2−3 nt 3′-overhang. Therefore, plant Hen1 appears to have acquired additional domains specifically for the recognition and association of small RNA duplexes of 21−24 nt, allowing efficient 3′-terminal 2′-O-methylation of small RNAs by the MTase domain.[11,18,19] In animals, the situation is slightly different. Unlike small RNAs in plants, siRNAs and miRNAs in most animals are not 2′-O-methylated at their 3′-terminal nucleotides. Instead, piRNAs, which are generated by Piwi proteins in the form of single-stranded RNAs of 24−31 nt, require 3′-terminal 2′-O-methylation. Animal Hen1 appears to have acquired a C-terminal domain for its association with Piwi proteins for the coordinated generation and 3′-terminal 2′-O-methylation of piRNAs.[13,14,25]

It is interesting to note that unlike Hen1, Dicer and Argonaute proteins, the other two essential enzymes responsible for RNAi in eukaryotic organisms, are highly conserved between plants and animals. Therefore, it is possible that in the early stages of evolution of the RNAi system, eukaryotic organisms might not have had Hen1 as part of the RNAi apparatus. Hen1 might have been acquired by or evolved at a later stage in eukaryotic organisms to enhance the stability of small RNAs via 3′-terminal 2′-O-methylation, thus increasing the efficiency of RNAi. The requirement for 3′-terminal 2-O-methylation of different types of small RNAs in plants and animals might have resulted in the existence of two different forms of Hen1 in these two different kingdoms of organisms.

The situation in bacteria is entirely different because bacteria have no RNAi. Instead, bacterial Hen1 was found to be involved in RNA repair.[12] Because of 3′-terminal 2′-O-methylation by Hen1 during RNA repair, the repaired RNA resists future RNA damage at the repair site. To maintain the quality of the repaired RNA (e.g., repaired RNAs are 2′-O-methylated), bacterial Pnkp/Hen1 systems have evolved in such a way that the ligation step of RNA repair requires the participation of Hen1. Such an arrangement ensures the opportunity of the MTase domain of Hen1 to conduct 2′-O-methylation during RNA repair. Our recent structural and biochemical studies of the bacterial Pnkp-C/Hen1-N heterodimer (Figure 4A, colored green) revealed that the N-terminal domain of bacterial Hen1 is an activator that turns on the ligase activity of Pnkp for RNA repair.[37] Therefore, bacterial Hen1 appears to have acquired an N-terminal domain to regulate the ligase activity of bacterial Pnkp for quality control of the repaired RNAs.

The discussions outlined above focused on the possible evolutionary acquisition of additional domains on top of the MTase domain in Hen1. A follow-up and intriguing question involves the origin of the MTase domain. If the acquisition of the biological functions of Hen1 is a relatively recent event over the course of evolution, it is then possible that the MTase domain of Hen1 might have evolved from another more ancient methyltransferase. One such scenario is presented here to demonstrate how the evolution could occur.

Bioinformatic analysis of Hen1 by Tkaczuk et al. showed that the MTase domain of Hen1 is distinct from other known RNA 2′-O-methyltransferases and is more similar to some small molecule methyltransferases.[28] Of particular relevance is the revelation in their two-dimensional cluster analysis that the cluster of bacterial Hen1 is closest in distance to the cluster of UbiG, a methyltransferase implicated in ubiquinone biosynthesis.[38] This was confirmed by our recent Blastp search using the sequence of the MTase domain of *Ava*Hen1 as the search model (data not shown). With the availability of the structure of *Ath*Hen1 in complex with RNA to pinpoint the precise amino acids involved in catalysis, it is worth revisiting the Hen1−UbiG relationship to shed some light on the possible evolutionary origin of the MTase domain of Hen1.

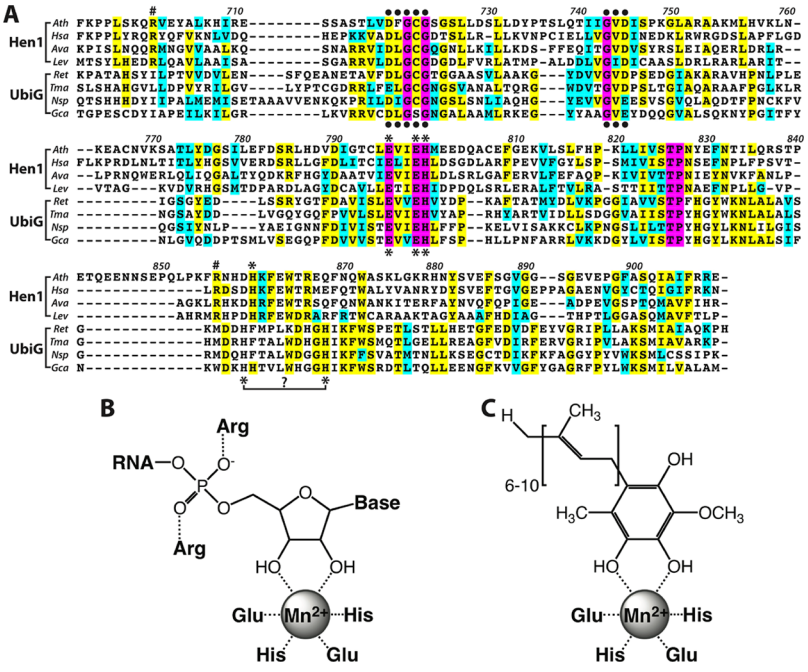

Figure 7. Proposed mechanistic similarities of O-methylation conducted by the MTase domain of Hen1 and UbiG. (A) Sequence alignments of the MTase domain of Hen1 and four representatives of UbiG. The residues are colored, numbered, and marked the same as the alignments shown in Figure 2B, except the marked conserved residues of Hen1 are on top and those of UbiG at the bottom of the alignments. Abbreviations: *Ret*, *Rhizobium etli*; *Tma*, *Thiocapsa marina*; *Nsp*, *Nodularia spumigena*; *Gca*, *Gallionella capsiferriformans*. (B) Chemical structure of the 3′-terminal nucleotide of RNA as the substrate of 2′-O-methylation by Hen1. The six conserved residues from Hen1 coordinating a metal ion or interacting with the phosphate backbone of the 3′-terminal nucleotide are schematically depicted. (C) Chemical structure of a precursor of ubiquinone biosynthesis that is the substrate of O-methylation by UbiG. The predicted coordinations of a metal ion by the side chains of four conserved residues from UbiG are also schematically depicted.

Among six strictly conserved residues in the MTase domain of Hen1 that are essential for catalysis (Figure 6), three of the four that are involved in coordinating a metal ion are concentrated on a short peptide motif of **E**XI**EH** (Figure 2B). This motif is conserved in UbiG as **E**XI/V**EH** (Figure 7A). Because of the conservation, the three conserved amino acids in the **E**XI/V**EH** motif of UbiG are predicted to also coordinate a metal ion for catalysis (Figure 7C). In *Ath*Hen1, H860 is the fourth amino acid coordinating the metal ion (Figure 7A). Although the equivalent residue in UbiG is not a histidine, two nearby conserved histidines in UbiG are good candidates to be the fourth ligand for the metal ion coordination by UbiG (Figure 7A, marked with a question mark). Consistent with lack of a phosphate group in the substrate of UbiG (Figure 7C), two essential arginines in Hen1 responsible for interacting with the phosphate backbone of the 3′-terminal nucleotide of RNA are not conserved in UbiG (Figure 7A). Therefore, if the MTase domain of Hen1 was evolved from an UbiG-like small molecule methyltransferase, one could speculate that a minimal requirement would be introduction of two arginines for interacting with the phosphate backbone of the 3′-terminal nucleotide of the RNA substrate.

■ CONCLUDING REMARKS

The presence of 2′-OH groups in RNA is the main reason RNA is susceptible to degradation in vivo. A 2′-OH group in RNA can conduct a nucleophilic attack on its adjacent phosphate backbone, resulting in RNA cleavage. Methylation of a 2′-OH group in an RNA increases the stability of the RNA by blocking the 2′-OH group as the nucleophile for RNA cleavage. At first glance, the argument described above does not apply to the 2′-OH group at the 3′-terminal nucleotide of RNA as it has no nearby cleavage target. However, a unique methyltransferase named Hen1 was discovered to conduct 2′-O-methylation specifically at the 3′-terminal nucleotide of RNA, and 2′-O-methylation results in stabilization of the targeted RNA. To date, the biological and biochemical functions of Hen1 from major species of living organisms have been well-defined on the basis of studies of Hen1 from plants, animals, and bacteria. Specifically, forms of Hen1 from plants, which are classified as the first subfamily, perform 3′-terminal 2′-O-methylation of the double-stranded siRNAs and miRNAs. Forms of Hen1 from most animals, which are classified as the second subfamily, conduct 3′-terminal 2′-O-methylation of the single-stranded piRNAs. The bacterial forms of Hen1 of the third subfamily conduct 3′-terminal 2′-O-methylation of the damaged RNA during RNA repair.

Despite the significant progress described above, several issues remain unresolved. Unlike forms of Hen1 from the first and third subfamilies,[19,37] no structural information about the C-terminal domain of Hen1 of the second subfamily is available. Therefore, the proposed interaction of the C-terminal domain of animal Hen1 with Piwi proteins requires further biochemical and structural confirmation. *Vca*Hen1, the only Hen1 from the organisms of the Chlorophyta phylum in the Viridiplantae kingdom, has no sequence similarities with *Ath*Hen1 beyond the MTase domain. Therefore, it is unclear whether the biochemical function of *Ath*Hen1, which belongs to the Streptophyta phylum, is conserved in Hen1 from the organisms of the Chlorophyta phylum. Addressing this issue requires more genomic sequencing of organisms belonging to the Chlorophyta phylum and biochemical and structural

dx.doi.org/10.1021/bi300497x | *Biochemistry* 2012, 51, 4087−4095

characterization of a Hen1 from an organism of the Chlorophyta phylum. Among three subfamilies of Hen1 possessing extra domains, forms of Hen1 of the second subfamily are the most diverse, illustrated by the distribution of forms of Hen1 in four different groups as well as the majority of species of the unclustered Hen1 in the sequence similarity network (Figure 1, groups 2 and 4−6 and others). Our current biochemical knowledge of forms of Hen1 of the second subfamily is based on studies of forms of Hen1 from animals. Therefore, determining whether the biochemical function of Hen1 is conserved in more distant species, such as those of Fungi, Euglenozoa, and Alveolata, requires further investigation. Finally, forms of Hen1 of the fourth subfamily, which only has the MTase domain, require experimental confirmation that the enzyme is capable of performing 3′-terminal 2′-O-methylation of RNA. Further studies are also required to shed some light on the biological functions of Hen1 of the fourth family.

Structural and biochemical studies of Hen1 revealed that forms of Hen1 conduct RNA 3′-terminal 2′-O-methylation via a metal ion-dependent mechanism. The requirement of 2′- and 3′-OH groups of the targeted nucleotide in RNA for coordination of the metal ion addresses the issue of substrate specificity as the nucleotide at the 3′-teminus of an RNA is the only one that has both OH groups. Despite the elegant solution by Hen1 for specific RNA 3′-terminal 2′-O-methylation, one cannot rule out the presence of another methyltransferase in living organisms that is also capable of conducting RNA 3′-terminal 2′-O-methylation. For example, a methyltransferase could employ the same metal ion-dependent mechanism as Hen1 but use a different strategy to provide four amino acids for metal ion coordination. Alternatively, a methyltransferase could employ a mechanism that does not require a metal ion as seen in other RNA 2′-O-methyltransferases but could utilize the steric difference of the 3′-terminal nucleotide from other nucleotides in RNA (less bulky 3′-OH group vs bulkier phosphate group) to achieve substrate specificity. Therefore, it remains to be seen if a methyltransferase other than Hen1 will be discovered in the future to conduct 2′-O-methylation at the 3′-terminal nucleotide of RNA.

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Funding

This project was supported by National Science Foundation Grant MCB-0920966.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

I am very grateful to Chio Mui Chan, Chun Zhou, Pei Wang, Can Zhang, and David Christensen for their contributions to the RNA repair project. I thank Kiruthika Selvadurai for critical reading of the manuscript.

ABBREVIATIONS

MTase, methyltransferase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; RNAi, RNA interference.

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