The Mammalian de Novo DNA Methyltransferases DNMT3A and DNMT3B Are Also DNA 5-Hydroxymethylcytosine Dehydroxymethylases*1

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Background: The pathways of DNA dehydroxymethylation and demethylation are yet to be better defined.

Results: De novo DNMTs could serve as redox state-dependent DNA dehydroxymethylases.

Conclusion: DNA dehydroxymethylation by DNMTs provides a simpler pathway to reduce DNA hydroxymethylation and methylation.

Significance: That de novo DNMTs also function as DNA dehydroxymethylases raises intriguing new questions regarding their structures and regulatory roles.

For cytosine (C) demethylation of vertebrate DNA, it is known that the TET proteins could convert 5-methyl C (5-mC) to 5-hydroxymethyl C (5-hmC). However, DNA dehydroxymethylase(s), or enzymes able to directly convert 5-hmC to C, have been elusive. We present in vitro evidence that the mammalian de novo DNA methyltransferases DNMT3A and DNMT3B, but not the maintenance enzyme DNMT1, are also redox-dependent DNA dehydroxymethylases. Significantly, intactness of the C methyltransfer catalytic sites of these de novo enzymes is also required for their 5-hmC dehydroxymethyl activity. That DNMT3A and DNMT3B function bidirectionally both as DNA methyltransferases and as dehydroxymethylases raises intriguing and new questions regarding the structural and functional aspects of these enzymes and their regulatory roles in the dynamic modifications of the vertebrate genomes during development, carcinogenesis, and gene regulation.

Methylation (1) and hydroxymethylation (2–4) at the C-5 position of cytosine (C) in the CpG dinucleotide context are the two major DNA modifications observed in vertebrate genomes. The roles of 5-methyl C (5-mC)3 in the epigenetic control of gene regulation, chromatin structure, development, and diseases are well documented (5–7), whereas those of the 5-hydroxymethyl C (5-hmC) are still being investigated. In contrast to our understanding of DNA methylation by the enzymatic activities of the vertebrate DNA methyltransferases (DNMTs), such as the de novo enzymes DNMT3A/DNMT3B and the maintenance enzyme DNMT1 (8–11), the processes of removing 5-mC or 5-hmC from DNA, i.e. DNA demethylation or DNA dehydroxymethylation, in vertebrate cells are debated.

More recently, it has been shown that 5-mC on DNA can be enzymatically converted to 5-hmC, 5-formylcytosine, and 5-carboxylcytosine (5-caC) by the TET (ten-eleven translocation) proteins or methylcytosine dioxygenases family of enzymes (3, 12–15). In vivo, the preferential genome-wide demethylation of the hypermethylated paternal genome at the zygote stage is considered to be the result of passive demethylation, which relies on a combination of DNA replication and DNMT repression (16, 17). Significantly, 5-mC residues on the paternal chromosomes have been found to be converted to 5-hmC ahead of DNA replication. These 5-hmC residues might contribute to DNA demethylation by interfering with the substrate recognition of DNMT1 (18, 19) or by serving as intermediates for active demethylation process(es) in the paternal pronucleus (20, 21). In addition, it has been proposed that active DNA demethylation in vertebrate cells, either on a genome-wide scale or at specific genomic loci, can be achieved by DNA repair-dependent processes removing the oxidation/deamination derivatives of 5-mC (13, 22–26). However, the extent of the involvement of the DNA repair machinery in the active DNA demethylation of the vertebrate genomes remains to be seen. In the following, we present in vitro evidence supporting that DNMT3A and DNMT3B could also function as active DNA 5-hmC dehydroxymethylases.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids, Proteins, and Antibodies—The control plasmid pCI-EGFP was constructed by shifting the EGFP fragment from pEFGP-C1 (Clontech) to the pCI expression vector (Promega). Plasmid DNAs were also constructed for the overexpression of mouse TET1, DNMT1, DNMT3A, and DNMT3B in 293T cells. The DNA inserts of these plasmids were cDNAs prepared from mouse ES cells and cloned into the pCI expression vector (Promega). The DNA methylation-inactive variant of DNMT1, DNMT1-PSC, was constructed by site-

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3 The abbreviations used are: 5-mC, 5-methyl C; 5-hmC, 5-hydroxymethyl C; 5-caC, 5-carboxylcytosine; DNMT, DNA methyltransferase; EGFP, enhanced green fluorescent protein; 2-ME, β-mercaptoethanol; 2-OG, 2-oxoglutarate; TET, ten-eleven translocation proteins; h, human.
directed mutagenesis inserting a serine before Cys-1229 of DNMT1 to mimic the inactive DNA methyltransferase homologue of fission yeast (27). To obtain the inactive counterparts of DNMT3A and DNMT3B, DNMT3A-PS and DNMT3B-PS, respectively, the cysteine residues in the methylation-active centers, Cys-706 of DNMT3A and Cys-657 of DNMT3B (8), were replaced with a serine residue. The recombinant DNMT proteins, including the human DNMT1 (purity ~78%), human DNMT3A (purity ~90%), and mouse DNMT3B (purity ~50%), were purchased from BPS Bioscience. The anti-DNMT1 antibody was purchased from Cosmo Bio. Anti-DNMT3A and anti-DNMT3B antibodies were purchased from Millipore.

Cell Culture, DNA Transfection, and Nuclear Extract Preparation—Human 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Biological Industries) and 1% penicillin-streptomycin (Invitrogen) under 5% CO2 at 37 °C. Approximately 3 × 10^6 cells were plated in the 10-cm culture dishes and transfected with 10 μg of the different expression plasmids using Lipofectamine 2000 following the standard procedure (Invitrogen). After 48 h, the cells were trypsinized and collected for further experiments. The nuclear extracts were prepared from 293T cells as described previously (28).

DNA Substrates—The double-stranded DNA substrates for the hydrolysis-TLC assay of 5-hmC to C or 5-mC to 5-hmC conversion were purchased from Diagenode. The 5-mC-containing substrate is 300 bp long, and the 5-hmC-containing substrate is 280 bp long. The 5-mC-containing substrate consisted of 78 residues of 5-mC, among which 18 were in the CpG context, and 1 methylated CCGG sequence (MspI/HpaII site). The 5-hmC-containing substrate consisted of 73 residues of 5-hmC, 12 of which were in the context of CpG, and 1 hydroxymethylated CCGG sequence (MspI/HpaII site). For restriction digestion-PCR assay of the 5-hmC to C conversion, the 5-hmC-containing DNA substrate was prepared by PCR amplification of a 501-bp fragment from the pMR1–8 plasmid containing MspI/HpaII sites. During the PCR amplification, a 5-hmC-containing dNTP mix (Zymo Research) was used. The 5-hmC substrate was recovered with the QIAquick PCR Purification kit (Qiagen), and its hydroxymethylation was confirmed by glycosylation with glucosyltransferase (New England Biolabs) followed by digestion with MspI (29). For in vitro DNA methylation assay, the unmodified pMR1–8 plasmid prepared from the SCS110 bacteria (Stratagene) was used as the DNA substrate.

In Vitro Reactions of 5-hmC to C Conversion on DNA—For the dehydroxymethylation reactions, 40 ng of one of two different 5-hmC DNA substrates was incubated at 37 °C for 1–4 h with 100 μg of the different nuclear extracts or 10–40 nM of the recombinant enzymes in 50 μl of buffer B (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1.5 mM MgCl2, and protease inhibitors (Roche Applied Science)) (28) containing 100 μg/ml BSA. When needed (see Fig. 1), 100 μM Fe2+, 1 mM 2-OG, or 1–5 mM DTT was also included in the reaction mixtures. To further investigate the role of the redox state of the enzyme in the conversion of 5-hmC to C, 40 nM of recombinant human DNMT3A (hDNMT3A) was preincubated with or without 1–5 mM DTT, 1–2 mM β-mercaptoethanol, or 1–10 mM H2O2 in 50 μl of buffer B at 15 °C for 30 min. After the preincubation, 40 ng of the 5-hmC DNA substrate was added, and the reaction mixtures were incubated at 37 °C for 2 h.

All reactions were stopped with 1.3% SDS and treated with proteinase K at 50 °C for 20 min. The DNA substrates were purified as described above and subjected to hydrolysis-TLC assay or restriction digestion-PCR assay to determine the extent of 5-hmC to C conversion.

In Vitro Reaction of C to 5-mC Conversion on DNA (DNA Methylation)—As a comparison with the 5-hmC to C reaction, the role of the redox state of hDNMT3A in the C to 5-mC conversion was also examined by pretreatment of 40 nM of the recombinant enzyme without or with 1–5 mM DTT, 1–2 mM β-mercaptoethanol, or 1–10 mM H2O2 in 50 μl of buffer B at 15 °C for 30 min. Unmodified pMR1–8 plasmid DNA (100 ng) was then incubated with the pretreated recombinant hDNMT3A in 50 μl of buffer B supplemented with 100 μg/ml BSA and 160 μM S-adenosylmethionine (New England Biolabs) at 37 °C for 2 h. After the incubation, the reactions were stopped by 1.3% SDS and treated with proteinase K at 50 °C for 20 min. The DNA samples were isolated by QIAquick nucleotide removal kit (Qiagen) and subjected to the analysis by the hydrolysis-TLC assay.

Hydrolysis-TLC (Thin Layer Chromatography) Assay of 5-mC, 5-hmC, and C on Double-stranded DNA—The procedure (supplemental Fig. S1A) followed those of published reports (3, 14). Briefly, the double-stranded DNA substrates were digested with 10 units of MspI (New England Biolabs) at 37 °C and then dephosphorylated with 10 units of calf intestinal phosphatase (New England Biolabs). The DNA samples were purified and 5′-end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs). The DNA substrates were extensively digested with snake venom phosphodiesterase ( Worthington) and DNase I (Roche Applied Science). The hydrolysates were spotted on the PEI cellulose plates (Merck) and developed in isobutyric acid:water:ammonia (66:18:3) for 16–18 h. Afterward, the plates were autoradiographed and analyzed by Alpha Imaging 2200 (Alpha Innotech Corp.).

Restriction Digestion-PCR assay of 5-hmC on Double-stranded DNA Substrate—To determine the extent of dehydroxymethylation of 5-hmC on DNA by this assay (supplemental Fig. S1B), 20 ng of the 5-hmC-containing DNA substrate with or without the in vitro dehydroxymethylation reactions was digested by HpaII (New England Biolabs) at 37 °C overnight and recovered with QIAquick nucleotide removal kit (Qiagen).
The proportion of uncut DNA substrate was quantitated by 16 cycles of PCR using a primer pair bracketing the HpaII sites followed by electrophoresis of the DNA products on agarose gel (see Fig. 2B) (30). The primer sequences used in PCR are available upon request.

RESULTS

DNA Dehydroxymethylation Activity of Mammalian DNMT3A—We sought other enzymatic pathway(s) that might contribute to the active demethylation of the vertebrate genome. To our surprise, we found that the mammalian DNMTs, in particular DNMT3A and DNMT3B, but not DNMT1, could effectively convert 5-hmC to C. In vitro oxidation assays were carried out using 5-hmC- or 5-mC-containing double-stranded DNA oligonucleotides incubated with nuclear extracts prepared from 293T cells transfected with plasmids expressing the mouse TET1 or DNMT3A, respectively. After extracts prepared from 293T cells transfected with plasmids overexpressing mouse TET1 or DNMT3A, respectively. After incubation with nuclear extracts from 293T cells the DNA substrates were hydrolyzed (supplemental Fig. S1) (33118).

In vitro dehydroxymethylation activities of mouse DNMT1, DNMT3A, and DNMT3B. The 5-hmC-containing DNA substrates were subjected to reactions in buffer B with 293T nuclear extracts containing the exogenously expressed EGFP and mouse DNMT1, DNMT3A, and DNMT3B in addition to their site-directed mutants. The extents of conversion of 5-hmC to C were analyzed by hydrolysis-TLC assay and quantitatively shown in the histograms. The level of the activity in EGFP-containing extract (lane 2) is similar to that of nuclear extract from untransfected 293T cells (data not shown). M, mock without incubation; R, with incubation. **, p < 0.01. Error bars indicate S.D.

Requirement of Intact DNA 5-mC Catalytic Sites of DNMT3A—DNMT3A was not the only DNMT capable of converting 5-hmC to C. We tested and compared the in vitro activities of 293T nuclear extracts containing exogenously expressed DNMT1, DNMT3A, and DNMT3B. The 5-hmC-containing DNA substrates resistant to HpaII cleavage were analyzed. The histogram shows the proportion of DNA substrates resistant to HpaII cleavage. **, p < 0.05; ***, p < 0.01. Error bars indicate S.D. which appeared to require the presence of reducing reagents such as DTT (10, 19).

In addition to the hydrolysis-TLC assay, we also used restriction digestion coupled with PCR (supplemental Fig. S1B) to confirm the 5-hmC dehydroxymethylation activities of DNMT3A and DNMT3B. In this assay, the 5-hmC-containing DNA substrates indeed became HpaII-sensitive after incuba-

FIGURE 1. DNA 5-hydroxymethylcytosine dehydroxymethylase activity of mouse DNMT3A. The 5-hmC or 5-mC-containing double-stranded DNA substrates were subjected to reactions with nuclear extracts from 293T cells overexpressing mouse TET1 (lanes 1–4) or DNMT3A (lanes 5–9), in buffer A (lanes 1 and 2) or buffer B (lanes 3–9) with or without the inclusion of 100 μM Fe^{2+}, 1 mM 2-OG, or 5 mM DTT. After the reactions, the DNA products were subjected to hydrolysis-TLC assay. The extents of conversion of 5-mC to 5-hmC and 5-hmC to C are shown in the two histograms. M, mock control without incubation. **, p < 0.01. Error bars indicate S.D.

FIGURE 2. DNA 5-hydroxymethylcytosine dehydroxymethylase activities of mammalian DNMT3A and DNMT3B. A, comparison of 5-hmC dehydroxymethylation activities of mouse DNMT1, DNMT3A, and DNMT3B. The 5-hmC-containing DNA substrates were subjected to reactions in buffer B with 293T nuclear extracts containing the exogenously expressed EGFP and mouse DNMT1, DNMT3A, and DNMT3B in addition to their site-directed mutants. The extents of conversion of 5-hmC to C were analyzed by hydrolysis-TLC assay and quantitatively shown in the histograms. The level of the activity in EGFP-containing extract (lane 2) is similar to that of nuclear extract from untransfected 293T cells (data not shown). M, mock without incubation; R, with incubation. **, p < 0.01. Error bars indicate S.D. DNMT1-PS, DNA methylation-inactive variant of DNMT1; DNMT3A-PS and DNMT3B-PS, inactive counterparts of DNMT3A and DNMT3B. B, 5-hmC dehydroxymethylase activities of DNMT1, DNMT3A, and DNMT3B by restriction digestion-PCR assay. The products after incubation of the double-stranded DNA substrates with the different 293T nuclear extracts in buffer B were analyzed. The histogram shows the proportion of DNA substrates resistant to HpaII cleavage. *p < 0.05; ***, p < 0.01. Error bars indicate S.D.
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FIGURE 3. Hydrolysis-TLC assay of the activities of the recombinant enzymes in the conversion of 5-hmC to C or conversion of C to 5-mC. A, hydrolysis-TLC assay of conversion of 5-hmC to C by recombinant hDNMT1, hDNMT3A, and DNMT3B. 5-hmC-containing DNA substrates were incubated with increasing concentrations (10, 20, 40 nM) of commercial recombinant hDNMT1, hDNMT3A, or mouse DNMT3B in buffer B and then analyzed. As exemplified for one plate and quantitatively shown in the histogram, only the hDNMT3A (lanes 6 – 8) and DNMT3B (lanes 9 – 11), but not hDNMT1 (lanes 3 – 5), function as DNA 5-hmC dehydroxymethylase. M, mock without incubation. B, hydrolysis-TLC assay of the effects of redox on the activities of recombinant hDNMT3A to convert 5-hmC to C or C to 5-mC. The DNA dehydroxymethylation reactions (lanes 1 – 8) or DNA methylation reactions (lanes 9 – 16) were carried out using appropriate DNA substrates and recombinant hDNMT3A preincubated with 1 mM DTT (lanes 3 and 11), 5 mM DTT (lanes 4 and 12), 1 mM 2-ME (lanes 5 and 13), 2 mM 2-ME (lanes 6 and 14), 1 mM H2O2 (lanes 7 and 15), or 10 mM H2O2 (lanes 8 and 16). The DNA samples were then analyzed on the TLC plate after hydrolysis and radioactive labeling. M, mock without incubation; R, reaction using the hDNMT3A without preincubation with the reducing or oxidizing reagents. Error bars indicate S.D.

FIGURE 4. Pathways of active methylation and demethylation of C in vertebrate DNA are shown. The known active pathways of C methylation by the three DNMTs, oxidation of 5-mC by TET, as well as removal of 5-caC and 5-hmC by DNA glycosylases/base excision repair/nextonucleotide excision repair (BER/NER) are indicated by the thin lines (see also under “Discussion” and see Refs. 25 and 26). The active conversion of 5-hmC to C by DNMT3A and DNMT3B, as revealed by this study, is indicated by the thick line. 5-IC, 5-formylcytosine.

DISCUSSION

Based on the data presented in Figs. 1–3 and supplemental Fig. S1, we suggest that the vertebrate de novo DNA methyltransferases DNMT3A and DNMT3B, but not the maintenance enzyme DNMT1, could also function as reduction-oxidation (redox) state-dependent “DNA dehydroxymethylases” (Fig. 4). It should be noted that the possibility of contaminating proteins in the extracts or commercial enzyme preparations, giving rise to the observed DNA dehydroxymethylase activity of DNMT3A/3B, could not be completely ruled out. However, the combined data of Figs. 2 and 3 strongly support the model that DNMT3A and -3B also function as DNA dehydroxymethylases.

This discovery of a new role of the vertebrate de novo DNMTs as active DNA 5-hmC dehydroxymethylases has several intriguing and important implications regarding the regulation of the vertebrate DNA modifications. First, the 5-hmC dehydroxymethylase activities of DNMT3A/3B provide a simple pathway for the direct conversion of 5-hmC to C. This pathway of 5-hmC to C is also relatively “safer” than the other pathways such as the base excision repair/nucleotide excision repair
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processes (Fig. 4), with respect to maintaining the integrity of the genomic DNA during dehydroxymethylation. Second, this pathway also provides an enzymatic route for the reversion of 5-hmC to 5-mC because DNMT3A/3B could convert 5-hmC to C and then remethylate the C to generate 5-mC. Thus, in collaboration with the TET proteins and DNMT1, the de novo DNMTs could dynamically regulate the modification status of C of the vertebrate DNA both on a genome-wide scale and at specific loci of the genome. Thirdly, by the above mechanism, the role of the DNMT3A/3B as redox state-dependent DNA dehydroxymethylases (Fig. 3B) may explain in part the aberrant DNA methylation patterns in cancer development. In cancer cells, aberrant epigenetic changes in DNA methylation, e.g. global DNA hypomethylation complicated by regional hypermethylation (31, 32) and up-regulated expression of the DNMTs including DNMT3A and DNMT3B (31, 33), are frequently observed. The environment of the cancer cells, in particular the presence of oxidative stresses, likely would facilitate 5-mC oxidation (18, 34) as well as the dehydroxymethylation activities of DNMT3A/3B, thus leading to a profound reduction of the 5-hmC level (35–37) and also causing global hypomethylation of the cancer genomes (31, 32). In this aspect, it seems like that the de novo DNMTs serve a protective function when cells are under oxidative stress.

Interesting questions remain regarding the structural and functional features of DNMT3A and -3B, in relation to the mechanisms of 5-hmC dehydroxymethylation, which might be similar to that proposed for the bacterial C-5 methyltransferase Hhal (38), and the effect of the reduction-oxidation state of the enzymes. It should be noted that changes of enzymatic activities or substrate specificities by redox conditions are not without precedents. For example, gain of function upon oxidation has been demonstrated for the bacterial transcription factor OxyR, which is accompanied with disulfide bond formation (39). Disulfide bond formation is also a key signal for the anti-oxidant activity of yeast Yap1 in response to oxidative stress (40). Significantly, the disulfide reductase and chaperone activities of plant AtTrx-h3 are regulated by the redox state of the enzyme in opposite ways (41). Also, the peroxidase and phospholipase A2 activities of the mammalian Prdx6 are reversely regulated by oxidation (42). Thus, in view of the available structural information of DNMT3A and DNMT3B, in particular those of the methyltransferase catalytic domain and the Cys-rich domain (43), we speculate that the redox conditions could affect the patterns of the oxidation states of specific Cys residues and/or Cys-disulfide bond formation of the two enzymes, and consequently their conformations and the substrate specificities. Structural and biochemical analysis of the two de novo enzymes, in comparison with DNMT1, will provide the molecular basis of the dual enzymatic functions of DNMT3A/3B as affected by the redox conditions. Finally, in view of the bilateral functions of the catalytic sites of DNMT3A and DNMT3B, it is also tempting to speculate here that under the appropriate conditions, these two enzymes might also be able to convert 5-formylcytosine and 5-caC directly to C.

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